

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup> :</b> C12N 15/00, A01H 1/00 C12N 5/00, A01H 5/10 // C12N 9/10	<b>A1</b>	<b>(11) International Publication Number:</b> WO 87/ 05629 <b>(43) International Publication Date:</b> 24 September 1987 (24.09.87)
<b>(21) International Application Number:</b> PCT/EP87/00141 <b>(22) International Filing Date:</b> 11 March 1987 (11.03.87)  <b>(31) Priority Application Numbers:</b> 86400521.0 (EP) 87400141.5 (EP) <b>(32) Priority Dates:</b> 11 March 1986 (11.03.86) 21 January 1987 (21.01.87) <b>(33) Priority Countries:</b> GB, et al.  <b>(71) Applicants (for all designated States except US):</b> PLANT GENETIC SYSTEMS N.V. [BE/BE]; Kunstlaan 46, B-1040 Brussels (BE). BIOGEN N.V. [NL/NL]; Pieterma A 1 15, Willemstad, Curaçao (AN). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> LEEMANS, Jan [BE/BE]; Ellebochten 38, B-9210 Heusden (BE). BOTTERMAN, Johan [BE/BE]; Eedstraat 37, B-9710 Zwijnaarde (BE). DE BLOCK, Marc [BE/BE]; Vogelhoeckstraat 32, B-9219 Gentbrugge (BE). THOMPSON, Charles [US/CH]; 19, chemin des Palettes, CH-1212 Grand-Lancy (CH). MOUYA, Rao [IN/CH]; 10, avenue Secheron, CH-1202 Genève (CH).	<b>(74) Agent:</b> GUTMANN, Ernest; S.C. Ernest Gutmann - Yves Plasseraud, 67, boulevard Haussmann, F-75008 Paris (FR).  <b>(81) Designated States:</b> AU, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CM (OAPI patent), DK, FI, GA (OAPI patent), HU, JP, KR, ML (OAPI patent), MR (OAPI patent), NO, SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> PLANT CELLS RESISTANT TO GLUTAMINE-SYNTHETASE INHIBITORS, MADE BY GENETIC ENGINEERING		
<b>(57) Abstract</b>  DNA fragment containing a determined gene, the expression of which inhibits the antibiotic and herbicidal effects of Bialaphos and related products. It also relates to recombinant vectors, containing such DNA fragment, which enable this protective gene to be introduced and expressed into cells and plant cells.		

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Plant cells resistant to glutamine-synthetase inhibitors, made by genetic engineering.

5

The invention relates to a process for protecting plant cells and plants against the action of glutamine synthetase inhibitors.

10

It also relates to applications of such process, particularly to the development of herbicide resistance into determined plants.

15

It relates further to non-biologically transformed plant cells and plants displaying resistance to glutamine synthetase inhibitors as well as to suitable DNA fragments and recombinants containing nucleotide sequences encoding resistance to glutamine synthetase inhibitors.

20

Glutamine synthetase (hereafter simply designated by GS) constitutes in most plants one of the essential enzymes for the development and life of plant cells. It is known that GS converts glutamate into glutamine. GS is involved in an efficient pathway (the only one known nowadays) in most plants for the detoxification of ammonia released by nitrate reduction, aminoacid degradation or photorespiration. Therefore potent inhibitors of GS are very toxic to plant cells. A particular class of herbicides has been developed, based on the toxic effect due to inhibit inhibition of GS in plants.

25

These herbicides comprise as active ingredient a GS inhibitor.

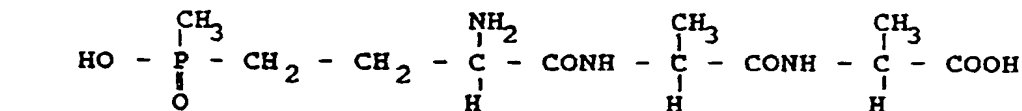
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There are at least two possible ways which might lead to plants resistant to the inhibitors of the action of glutamine synthetase ; (1) by changing the target. It can be envisaged that mutations in the GS enzyme can lead to insensitivity towards the herbicide ; (2) by inactivation of the herbicide. Breakdown or modification of the herbicide inside the plant could lead to resistance.

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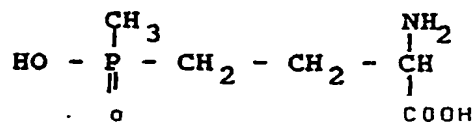
Bialaphos and phosphinothricin (hereafter simply designated by PPT) are two such inhibitors of the action of GS, (ref. 16, 17) and have been shown to possess excellent herbicidal properties (see more particularly  
 5 ref. 2 as concerns Bialaphos).

Bialaphos has the following formula (I) :



10

PPT has the following formula (II) :



15

Thus the structural difference between PPT and Bialaphos resides in the absence of two alanine aminoacids in the case of PPT.

These two herbicides are non selective. They inhibit growth of all the different species of plants present on the soil, accordingly cause their total destruction.

20

Bialaphos was first disclosed as having antibiotic properties, which enabled it to be used as a pesticide or a fungicide. Bialaphos can be produced according to the process disclosed in united-states patent n° 3 832 394, assigned to MEIJI SEIKA KAISHA LTD., which patent is incorporated herein by reference. It comprises cultivating  
 25 Streptomyces hygroscopicus, such as the strain available at the American Type Culture Collection, under the ATCC number 21,705, and recovering Bialaphos from its culture medium. However, other strains, such as Streptomyces  
 30 viridochromogenes, also produce this compound (ref. 1).

Other tripeptide antibiotics which contain a PPT moiety are or might be discovered in nature as well, e.g. phosalacin (ref. 15).

35 PPT is also obtained by chemical synthesis and is commercially distributed by the industrial Company

## HOECHST.

5 A number of Streptomyces species have been disclosed which produce highly active antibiotics which are known to incapacitate procaryotic cell functions or enzymes. The Streptomyces species which produce these antibiotics would themselves be destroyed if they had not a self defence mechanism against these antibiotics. This self defence mechanism has been found in several instances to comprise an enzyme capable of inhibiting the antibiotic effect, thus of avoiding autotoxicity for the Streptomyces species concerned. This modification is generally reversed when the molecule is exported from the cell.

15 The existence of a gene which encodes an enzyme able to modify the antibiotic so as to inhibit the antibiotic effect against the host has been demonstrated in several Streptomyces producing antibiotics, for example, in S. fradiae, S. azureus, S. vinaceus, S. erythreus, producing neomycin, thiostrepton, viomycin, and MLS (Macrolide Lincosamide Streptogramin) antibiotics respectively (ref. 4), (ref. 5), (ref. 6), (ref. 14 by CHATER et al., 1982 describes standard techniques which can be used for bringing these effects to light).

25 In accordance with the present invention, it has been found that Streptomyces hygroscopicus ATCC 21,705, also possesses a gene encoding an enzyme responsible of the inactivation of the antibiotic properties of Bialaphos. Experiments carried out by the applicants have lead to the isolation of such a gene and its use in a process for controlling the action of GS inhibitors, based on PPT or derived products.

30 An object of the invention is to provide a new process for controlling the action in plant cells and plants of GS inhibitors.

35 Another object of the invention is to provide DNA

fragments and DNA recombinants, particularly modified vectors containing said DNA fragments, which DNA fragments contain nucleotide sequences capable, when incorporated in plant cells and plants, to protect them against the action of GS inhibitors.

A further object of the invention is to provide non-biologically transformed plant cells and plants capable of neutralizing or inactivating GS inhibitors.

A further object of the invention is to provide a process for selectively protecting plant species against herbicides of a GS inhibitor type.

More specifically an object of the invention is to provide a DNA fragment transferable to plant cells- and to whole plants- capable of protecting them against the herbicidal effects of Bialaphos and of structurally analogous herbicides.

A further object of the invention is to provide plant cells resistant to the products of the class exemplified by Bialaphos, which products possess the PPT unit in their structure.

The process according to the invention for controlling the action in plant cells and plants of a GS inhibitor when contacted therewith, comprises providing said plants with a heterologous DNA fragment including a foreign nucleotide sequence, capable of being expressed in the form of a protein in said plant cells and plants, under condition such as to cause said heterologous DNA fragment to be integrated stably through generations in the cells of said plants, and wherein said protein has an enzymatic activity capable of inactivating or neutralization of said glutamine synthetase inhibitor.

A preferred DNA fragment is one derived from an antibiotic-producing-Streptomyces strain (or a sequence comprising a nucleotide sequence encoding the same activity) and which encodes resistance to a said GS



inhibitors.

Preferred nucleotide sequences for use in this invention encode a protein which has acetyl tranferase activity with respect to said GS inhibitors.

A most preferred DNA fragment according to the invention comprises a nucleotide sequence coding for a polypeptide having a PPT acetyl transferase activity.

A particular DNA fragment according to the invention, for the subsequent transformation of plant cells, consists of a nucleotide sequence coding for at least part of a polypeptide having the following sequence :

X SER PRO GLU  
183  
15 ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO  
229  
ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL  
273  
20 ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP  
318  
LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL  
363  
ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA  
409  
25 ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER  
453  
PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS  
498  
LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA  
543  
30 VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA  
588  
LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS  
633  
35 HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER  
673  
LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE  
723

6

in which X represents MET or VAL, which part of said polypeptide is of sufficient length to confer protection against Bialaphos to plant cells, when incorporated genetically and expressed therein, i.e. as termed  
 5 hereafter "plant-protecting capability" against Bialaphos.

A preferred DNA fragment consists of the following nucleotide sequence :

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10      183      GTG AGC CCA GAA
      CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
      228
      GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
      273
15      AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
      318
      CTC GTC CGT CTG CCG GAG CCG TAT CCC TGG CTC GTC GCC GAG GTG
      363
      GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
      408
20      CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
      453
      CCC CGC CAC CAG CCG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
      498
25      CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
      543
      GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CCG ATG CAC GAG GCG
      588
      CTC GGA TAT GCC CCC CGC GGC ATG CTG CCG GCG GCC GGC TTC AAG
      633
30      CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
      678
      CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
      723

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35 or of a part thereof expressing a polypeptide having

plant-protecting capability against Bialaphos.

5 The invention also relates to any DNA fragment differing from the preferred one indicated hereabove by the replacement of any of its nucleotides by others, yet without modifying the genetic information of the preferred DNA sequence mentioned hereabove (normally within the meaning of the universal genetic code), and furthermore to any equivalent DNA sequence which would encode a polypeptide having the same properties, particularly a Bialaphos-resistance-activity.

10 It will be understood that the man skilled in the art should be capable of readily assessing those parts of the nucleotide sequences that could be removed from either side of any of the DNA fragments according to the invention, for instance by removing terminal parts on either side of said DNA fragment, such as by an exonucleolytic enzyme, for instance Bal31, by recloning the remaining fragment in a suitable plasmid and by assaying the capacity of the modified plasmid to transform appropriate cells and to protect it against the Bialaphos antibiotic or herbicide as disclosed later, whichever assay is appropriate.

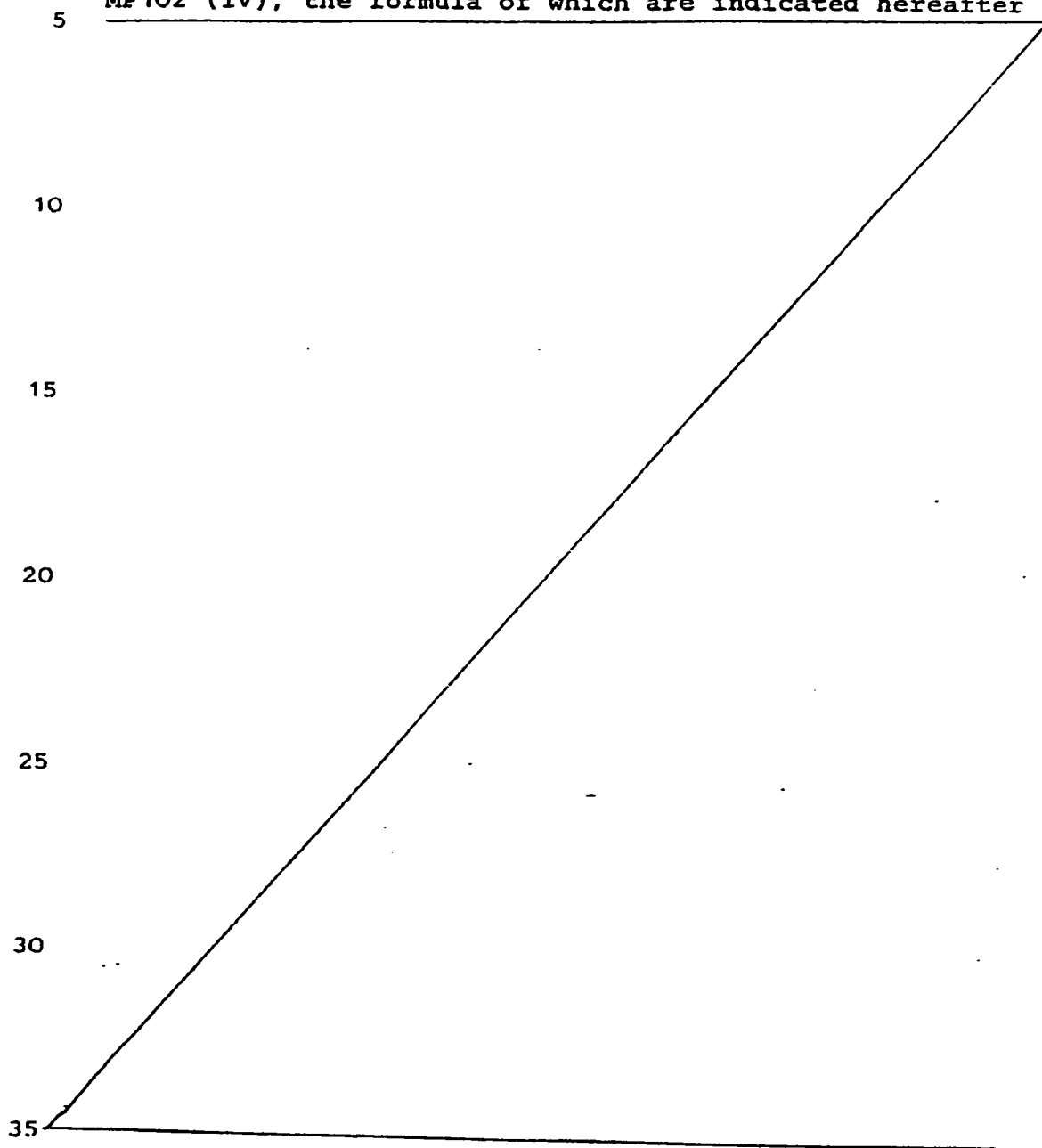
20 For the easiness of language, these DNA fragments will be termed hereafter as "Bialaphos-resistance DNA". In a similar manner, the corresponding polypeptide will be termed as "Bialaphos-resistance enzyme".

30 While in the preceding discussion particular emphasis has been put on DNA fragments capable, when introduced into plant cells and plants, to confer on them protection against Bialaphos or PPT, it should be understood that the invention should in no way be deemed as limited thereto.

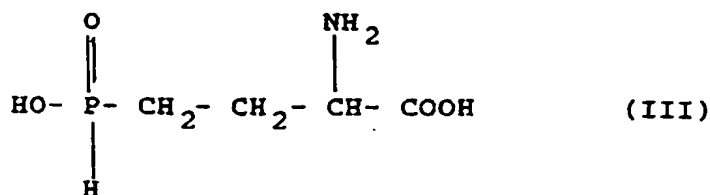
35 In a same manner, the invention pertains to DNA fragments which, when introduced into such plant cells, would also confer on them a protection against other GS

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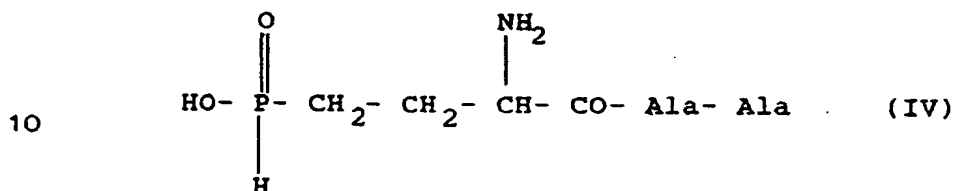
inhibitors, for instance of intermediate products involved in the natural biosynthesis of phosphinotricin, such as the compounds designated by the abbreviations MP101 (III), MP102 (IV), the formula of which are indicated hereafter :



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15 More generally, the invention has opened the route to the production of DNA fragments which, upon proper incorporation into plant cells and plants, can protect them against GS inhibitors when contacted therewith, as this will be shown in a detailed manner in relation to Bialaphos and PPT in the examples which will follow.

20 This having been established, it will be appreciated that any fragment encoding an enzymatic activity which would protect plant cells and plants against said GS inhibitors, by inactivation, should be viewed as an equivalent of the preferred fragments which have been disclosed hereabove. This would apply especially
 25 to any DNA fragments that would result from genetic screening of the genomic DNAs of strains, particularly of antibiotic-producing strains, likely to possess genes which, even though structurally different, would encode similar activity with respect to Bialaphos or PPT, or even
 30 with respect to other GS inhibitors. This applies to any gene in other strains producing a PPT derivative.

35 Therefore, it should be understood that the language "Bialaphos-resistance DNA" or "Bialaphos-resistance enzyme" used thereafter as a matter of convenience is intended to relate not only to the DNAs and

enzymes specifically concerned with resistance to PPT or most directly related derivatives, but more generally with other DNAs and enzymes which would be capable, under the same circumstances, of inactivating the action in plants of GS inhibitors.

The invention also relates to DNA recombinants containing the above defined Bialaphos-resistance DNA fragments recombined with heterologous DNA, said heterologous DNA containing regulation elements and said Bialaphos-resistance DNA being under the control of said regulation elements in such manner as to be expressible in a foreign cellular environment compatible with said regulation elements. Particularly the abovesaid Bialaphos-resistance-DNA fragments contained in said DNA recombinants are devoid of any DNA region involved in the biosynthesis of Bialaphos, when said Bialaphos-resistance-DNA fragment originate themselves from Bialaphos-producing strains.

By "heterologous DNA" is meant a DNA of an other origin than that from which said Bialaphos-resistance-DNA originated, e.g. is different from that of a Streptomyces hygroscopicus or Streptomyces viridochromogenes or even more preferably a DNA foreign to Streptomyces DNA. Particularly said regulation elements are those which are capable of controlling the transcription and translation of DNA sequences normally associated with them in said foreign environment. "Cellular" refers both to micro-organisms and to cell cultures.

This heterologous DNA may be a bacterial DNA, particularly when it is desired to produce a large amount of the recombinant DNA, such as for amplification purposes. In that respect a preferred heterologous DNA consists of DNA of E. coli or of DNA compatible with E. coli. It may be DNA of the same origin as that of the cells concerned or other DNA, for instance viral or plasmidic DNA known as

capable of replicating in the cells concerned.

Preferred recombinant DNA contains heterologous DNA compatible with plant cells, particularly Ti-plasmid DNA.

5 Particularly preferred recombinants are those which contain GS inhibitor inactivating DNA under the control of a promoter recognized by plant cells, particularly those plant cells on which inactivation of GS inhibitors is to be conferred.

10 Preferred recombinants according to the invention further relate to modified vectors, particularly plasmids, containing said GS-inhibitor-inactivating DNA so positioned with respect to regulation elements, including particularly promoter elements, that they enable said GS inhibitor-inactivating DNA to be transcribed and translated in the cellular environment which is compatible with said heterologous DNA. Advantageous vectors are those so engineered as to cause stable incorporation of said GS inhibitor inactivating DNA in foreign cells, particularly in their genomic DNA. Preferred modified vectors are those which enable the stable transformation of plant cells and which confer to the corresponding cells, the capability of inactivating GS inhibitors.

25 It seems that, as described later, the initiation codon of the Bialaphos-resistance-gene of the Streptomyces hygroscopicus strain used herein is a GTG codon. But in preferred recombinant DNAs or vectors, the Bialaphos-resistance-gene is modified by substitution of an ATG initiation codon for the initiation codon GTG, which ATG enables translation initiation in plant cells.

30 In the example which follows, the plant promoter sequence which has been used was constituted by a promoter of the 35 S cauliflower mosaic virus. Needless to say that the man skilled in the art will be capable of selecting other plant promoters, when more appropriate in relation

to the plant species concerned.

According to an other preferred embodiment of the invention, particularly when it is desired to achieve transport of the enzyme encoded by the Bialaphos-resistance-DNA into the chloroplasts, the heterologous DNA fragment is fused to a gene or DNA fragment encoding a transit peptide, said last mentioned fragment being then intercalated between the GS inhibitor inactivating gene and the plant promoter selected.

As concerns means capable of achieving such constructions, reference can be made to the following British applications 84 32757 filed on December 28, 1984 and 85 00336 filed on January 7, 1985 and to the related applications filed in the United-States of America (n° 755,173, filed on July 15, 1985), in the European Patent Office (n° 85 402596.2, filed on December 20, 1985), in Japan (n° 299 730, filed on December 27, 1985), in Israel (n° 77 466 filed on December 27, 1985) and in Australia (n° 5 165 485, filed on December 24, 1985), all of which are incorporated herein by reference.

Reference can also be made to the scientific literature, particularly to the following articles :

- VAN DEN BROECK et al., 1985, Nature, 313, 358-363 ;
- SCHREIER and al., Embo. J., vol. 4, n° 1, 25-32.

These articles are also incorporated herein by reference.

For the sake of the record, be it recalled here that under the expression "transit peptide", one refers to a polypeptide fragment which is normally associated with a chloroplast protein or a chloroplast protein sub-unit in a precursor protein encoded by plant cell nuclear DNA. The transit peptide then separates from the chloroplast protein or is proteolitically removed, during the translocation process of the latter protein into the chloroplasts.



Examples of suitable transit peptides are those associated with the small subunit of ribulose-1,5 biphosphate (RuBP) carboxylase or that associated with the chlorophyl a/b binding proteins.

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There is thus provided DNA fragments and DNA recombinants which are suitable for use in the process defined hereafter.

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More particularly the invention also relates to a process, which can be generally defined as a process for producing plants and reproduction material of said plants including a heterologous genetic material stably integrated therein and capable of being expressed in said plants or reproduction material in the form of a protein capable of inactivating or neutralizing the activity of a glutamine synthetase-inhibitor, comprising the non biological steps of producing plants cells or plant tissue including said heterologous genetic material from starting plant cells or plant tissue not able to express that inhibiting or neutralizing activity, regenerating plants or reproduction material of said plants or both from said plant cells or plant tissue including said genetic material and, optionally, biologically replicating said last mentioned plants or reproduction material or both, wherein said non-biological steps of producing said plant cells or plant tissue including said heterologous genetic material, comprises transforming said starting plant cells or plant tissue with a DNA-recombinant containing a nucleotide sequence encoding said protein, as well as the regulatory elements selected among those which are capable of enabling the expression of said nucleotide sequence in said plant cells or plant tissue, and to cause the stable integration of said nucleotide sequence in said plant cells and tissue, as well as in the plant and reproduction material processed therefrom throughout generations.

The invention also relates to the cell cultures

containing Bialaphos-resistance-DNA, or more generally said GS-inhibitor-inactivating DNA, which cell cultures have the property of being resistant to a composition containing a GS inhibitor, when cultured in a medium  
5 containing a such composition at dosages which would be destructive for non transformed cells.

The invention concerns more particularly those plant cells or cell cultures in which the Bialaphos-resistance DNA is stably integrated and which  
10 remains present over successive generations of said plant cells. Thus the resistance to a GS inhibitor, more particularly Bialaphos or PPT, can also be considered as a way of characterizing the plant cells of this invention.

Optionally one may also resort to hybridization experiments between the genomic DNA obtained from said  
15 plant cells with a probe containing a GS inhibitor inactivating DNA sequence.

More generally the invention relates to plant cells, reproduction material, particularly seeds, as well  
20 as plants containing a foreign or heterologous DNA fragment stably integrated in their respective genomic DNAs, said fragments being transferred throughout generations of such plant cells, reproduction material, seeds and plants, wherein said DNA fragment encodes a  
25 protein inducing a non-variety-specific enzymatic activity capable of inactivating or neutralizing GS inhibitors, particularly Bialaphos and PPT, more particularly to confer on said plant cells, reproduction material, seeds and plants a corresponding non-variety-specific phenotype  
30 of resistance to GS inhibitors.

"Non-variety-specific" enzymatic activity or phenotype aims at referring to the fact that they are not characteristic of specific plant genes or species as this  
35 will be illustrated in a non-limitative way by the examples which will follow. They are induced in said plant

materials by essentially non-biological processes applicable to plants belonging to species normally unrelated with one another and comprising the incorporation into said plant material of heterologous DNA, e.g. bacterial DNA or chemically synthesized DNA, which does not normally occur in said plant material or which normally cannot be incorporated therein by natural breeding processes, and which yet confers a common phenotype (e.g. herbicide resistance) to them.

The invention is of particular advantageous use in processes for protecting field-cultivated plant species against weeds, which processes comprise the step of treating the field with an herbicide, e.g. Bialaphos or PPT in a dosage effective to kill said weeds, wherein the cultivated plant species then contains in their genome a DNA fragment encoding a protein having an enzymatic activity capable of neutralizing or inactivating said GS inhibitor.

By way of illustration only, effective doses for use in the abovesaid process range from about 0.4 to about 1.6 kg/Hectare of Bialaphos or PPT.

There follows now a disclosure of how the preferred DNA fragment described hereabove was isolated starting from the Streptomyces hygroscopicus strain available at the American Type Culture Collection under deposition number ATCC 21 705, by way of exemplification only.

The following disclosure also provides the technique which can be applied to other strains producing compounds with a PPT moiety.

The disclosure will then be completed with the description of the insertion of a preferred DNA fragment conferring to the transformed cells the capability of inactivating Bialaphos and PPT. Thus the Bialaphos-inactivating-DNA fragment designated thereafter by

Bialaphos-resistance gene or "sfr" gene, isolated by the above described technique into plasmids which can be used for transforming plant cells and conferring to them a resistance against Bialaphos, also merely by way of example for non-limitative illustration purposes.

The following disclosure is made with reference to the drawings in which :

- fig. 1 is a restriction map of a plasmid containing a Streptomyces hygroscopicus DNA fragment encoding Bialaphos-resistance, which plasmid, designated hereafter as pBG1 has been constructed according to the disclosure which follows ;

- fig. 2 shows the nucleotide sequence of a smaller fragment obtained from pBG1, subcloned into another plasmid (pBG39) and containing the resistance gene ;

- fig. 3 shows the construction of a series of plasmids given by way of example, which plasmids aim at providing suitable adaptation means for the insertion therein of the Bialaphos-resistance gene or "sfr" gene ;

- fig. 4A and 4B show the construction of a series of plasmids given by way of example, which plasmids contain suitable plant cell promoter sequences able to initiate transcription and expression of the foreign gene inserted under their control into said plasmids ;

- fig. 5A shows a determined fragment of the nucleotide sequence of the plasmid obtained in figure 3 ;

- fig. 5B shows the reconstruction of the first codons of a Bialaphos-resistance gene, from a FokI/BglII fragment obtained from pBG39 and the substitution of an ATG initiation codon for the GTG initiation codon of the natural "sfr" gene ;

- fig. 5C shows the reconstruction of the entire "sfr" gene, namely the last codons thereof, and its insertion into a plasmid obtained in figures 4A and 4B ;

- fig. 6A shows an expression vector containing

the "sfr" gene placed under the control of a plant cell promoter ;

- fig. 6B shows another expression vector deriving from the one shown in fig. 6A, by the substitution of some nucleotides.

- fig. 7 shows the construction of a series of plasmids given by way of examples, to ultimately produce plasmids containing the promoter region and the transit peptide sequence of a determined plant cell gene, for the insertion of the "sfr" gene under the control of said promoter region and downstream of said transit peptide sequence.

- fig. 8 to 11 will be referred to hereafter.

The following experiment was set up to isolate a Bialaphos-resistance-gene from S. hygroscopicus, according to standard techniques for cloning into Streptomyces.

2.5 µg of S. hygroscopicus genomic DNA and 0.5 µg of Streptomyces vector pIJ61 were cleaved with PstI according to the method described in ref. 6. The vector fragments and genomic fragments were mixed and ligated (4 hours at 10°C followed by 72 hours at 4°C in ligation salts which contain 66 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol and 0.1 mM ATP) at a total DNA concentration of 40 µg ml<sup>-1</sup> with T4 DNA ligase. Ligation products were introduced into 3 x 10<sup>9</sup> S. lividans strain 66 protoplasts by a transformation procedure mediated by polyethylene-glycol (PEG) as described hereafter. These protoplasts gave rise to 5 x 10<sup>7</sup> colonies and 4 x 10<sup>4</sup> pocks after regeneration on 20 plates of R2 agar containing 0.5 % of Difco yeast extract (R2 YE). Preparation and composition of the different mediums and buffers used in the disclosed experiments are described herein-after. When these lawns were replica-plated on minimal

medium plates containing 50  $\mu\text{g ml}^{-1}$  Bialaphos, drug resistant colonies appeared at a frequency of 1 per  $10^4$  transformants. After purification of the drug resistant colonies, there plasmid DNA was isolated and used to retransform S. lividans protoplasts. Non selective regeneration followed by replication to Bialaphos-containing-medium demonstrated a 100 % correlation between pocks and Bialaphos resistant growth. The recombinant plasmids of several resistant clones all contained a 1.7 Kb PstI insert (see fig. 1).

#### Subcloning of the herbicide resistance gene

The 1.7 Kb PstI insert was then subcloned into the high copy number streptomycete vector pIJ385 to generate plasmid pBG20. S. lividans strains which contained pBG20 were more than 500 times more resistant to Bialaphos.

S. lividans growth is normally inhibited in minimal medium containing 1  $\mu\text{g/ml}$  Bialaphos ; growth of transformants containing pBG20 was not noticeably inhibited in a medium containing 500  $\mu\text{g/ml}$  Bialaphos. The PstI fragment was also subcloned in either orientation into the PstI site of the plasmid pBR322, to produce plasmids pBG1 and pBG2, according to their orientation. A test on minimal M9 medium demonstrated that E. coli E8767 containing pBG1 or pBG2 was resistant to Bialaphos.

A  $\pm$  1.65 Kb PstI - BamHI fragment was subcloned from pBG1 into the plasmid pUC19 to produce the plasmid pBG39, and conferred Bialaphos resistance to E. coli, W3110, C600 and JM83.

Using an in vitro coupled transcription-translation system (ref. 5) from S. lividans extracts, the 1,65 Kb PstI - BamHI fragment in pBG39 was shown to direct the synthesis of a 22 Kd protein. In the following, this 1,65 Kb insert includes a fragment coding for a 22 Kd protein and will be called "sfr" gene.

Fine mapping and sequencing of the gene

A 625 bp Sau3A fragment was subcloned from pBG39 into pUC19 and still conferred Bialaphos resistance to a E. coli W3110 host. The resulting clones were pBG93 and pBG94, according to the orientation.

The orientation of the gene in the Sau3A fragment was indicated by experiments which have shown that Bialaphos resistance could be induced with IPTG from the pUC19 lac promoter in pBG93. In the presence of IPTG (0.5 mM) the resistance of pBG93/W3110 increased from 5 to 50 µg/ml on a M9 medium containing Bialaphos. The W3110 host devoid of pBG93, did not grow on M9 medium containing 5 µg/ml Bialaphos. These experiments demonstrated that the Sau3A fragment could be subcloned without loss of activity. They also provided for the proper orientation as shown in the fig. 2, enclosed thereafter. The protein encoded by these clones was detected by using coupled transcription-translation systems derived from extracts of S. lividans (ref. 7). Depending on the orientation of the Sau3A fragment, translation products of different sizes were observed ; 22 Kd for pBG94 and ± 28 Kd for pBG93. This indicated that the Sau3A fragment did not contain the entire resistance gene and that a fusion protein was formed which included a polypeptide sequence resulting from the translation of a pUC19 sequence.

In order to obtain large amounts of the protein, a 1.7 Kb PstI fragment from pBG1 was cloned into the high copy number Streptomyces replicon pIJ385. The obtained plasmid, pBG20, was used to transform S. hygroscopicus. Transformants which contained this plasmid had more than 5 times as much PPT acetylating activity and also had increased amounts of a 22 kd protein on sodium dodecyl-sulfate gels (SDS gels). Furthermore, both the acetyl transferase and the 22 kd protein appeared when the production of Bialaphos begun. The correlation of the in

vitro data, kinetics of synthesis, and amplified expression associated with pBG20 transformants strongly implied that this 22 Kd band was the gene product.

5 The complete nucleotide sequence of the 625 bp Sau3A fragment was determined as well as of flanking sequences. Computer analysis revealed the presence of an open reading frame over the entire length of the Sau3A fragment.

#### 10 Characterization of the sfr gene product

A series of experiments were performed to determine that the open reading frame of the "sfr" gene indeed encoded the Bialaphos resistance enzyme. To determine the 5' end of the resistance gene, the NH<sub>2</sub>-terminal sequence of the enzyme was determined. As concerns more particularly the technique used to determine the said sequence, reference is made to the technique developed by J. VANDEKERCKHOVE, Eur. J. Bioc. 152, p. 9-19, 1985, and to French patent applications n° 85 14579 filed on October 1st, 1985 and n° 85 13046 filed on September 2nd, 1985, all of which are incorporated herein by reference.

15 This technique allows the immobilization on glass fibre sheets coated with the polyquaternary amine commercially available under the registered trademark POLYBRENE of proteins and of nucleic acids previously separated on a sodium dodecylsulfate containing polyacrylamide gel. The transfer is carried out essentially as for the protein blotting on nitrocellulose membranes (ref. 8). This allows the determination of amino-acid composition and partial sequence of the immobilized proteins. The portion of the sheet carrying the immobilized 22 kd protein produced by S. hygrosopicus pBG20 was cut out and the disc was mounted in the reaction chambre of a gas-phase sequenator to subject the glass-fibre bound 22 Kd protein to the Edman degradation procedure. The following amino-acid sequence was obtained :

25  
30  
35



Pro-Glu-Arg-Arg-Pro-Ala-Asp-Ile-Arg-Arg

This sequence matched an amino-acid sequence which was deduced from the open reading frame of the 625 bp Sau3A fragment. It corresponded to the stretch from codon 3 to codon 12.

Thus, the  $\text{NH}_2$ -terminus of the 22 Kd protein was upstream of this sequence. It was determined that translation of the actual protein was likely to be initiated 2 amino-acids earlier at a GTG initiation codon. GTG is often used as initiator codon in *Streptomyces* and translated as methionine. The protein translated from the GTG initiation codon would be 183 amino-acids long and would have a molecular weight of 20 550. This was in good agreement with the observed approximate molecular weight of 22 000.

Furthermore, the termination codon, TGA, was located just downstream of the Sau3A site. Cloning of the 625 bp Sau3A fragment in a BamHI site digested pUC19 did not result in the reconstruction of the termination codon. This explained the fusion proteins which were observed in the in vitro transcription-translation analysis.

#### Mechanism of PPT-resistance

Having defined a first phenotype and some of the physical characteristics of the resistance gene and its gene product, a series of experiments was then carried out to understand the mechanism by which it confers resistance. As described hereabove, PPT is the portion of Bialaphos which inhibits glutamine synthetase (GS) and that N-acetyl PPT is not an inhibitor. Using a standard assay (ref. 9), S. hygrosopicus ATCC 21 705 derivatives were shown to contain a PPT acetyl transferase which was not found in S. lividans. The activity does not acetylate the Bialaphos tripeptide. S. lividans carrying the resistance gene cloned in pBG20 or pBG16 (a plasmid containing the 625 bp Sau3A fragment cloned into another

streptomycete vector, pIJ680) also contained the activity which could acetylate PPT but not Bialaphos. The PPT derived reaction product produced by extracts of pBG20/

5 S. lividans was isolated in order to confirm that it was indeed acetyl-PPT. Analysis by mass spectroscopy showed that the molecular weight had increased relative to PPT by the equivalent of one acetyl group. It was thus concluded that the 625 bp Sau3A fragment contained sequences which

10 code for PPT acetyl transferase.

The experimental conditions and reagents used in the techniques disclosed hereabove were as follows :

Preparation and composition of the mediums and buffers above used

15 1° P medium : 10.3 g of sucrose, 0.025 g of  $K_2SO_4$ , 0.203 g of  $MgCl_2 \cdot 6H_2O$  and 0.2 ml of a trace element solution are dissolved in 80 ml of distilled water and autoclaved. Then in order, 1 ml of  $KH_2PO_4$  (0.5 %), 10 ml of  $CaCl_2 \cdot 2H_2O$  (3.68 %), and 10 ml of TES buffer (0.25 M), pH : 7.2) are added. Trace element solution (per litre) :

20  $ZnCl_2$ , 40 mg ;  $FeCl_3 \cdot 6H_2O$ , 200 mg ;  $CuCl_2 \cdot 2H_2O$ , 10 mg ;  $MnCl_2 \cdot 4H_2O$ , 10 mg ;  $Na_2B_4O_7 \cdot 10H_2O$ , 10 mg ;  $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ , 10 mg.

25 2° R2YE : 10.3 g of sucrose, 0.025 g of  $K_2SO_4$ , 1.012 g of  $MgCl_2 \cdot 6H_2O$ , 1 g of glucose, 0.01 g of Difco casamino acids, and 2.2 g of Difco agar are dissolved in 80 ml distilled water and autoclaved. 0.2 ml of trace element solution, 1 ml of  $KH_2PO_4$  (0.5 %), 8.02 ml of  $CaCl_2 \cdot 2H_2O$  (3.68 %), 1.5 ml of L-proline (20 %), 10 ml of TES buffer (0.25 M) (pH : 7.2), 0.5 ml of (1 M) NaOH, 5 ml

30 of yeast extract (10 %) are sequentially added.

3° TE : 10 mM TRIS HCl, 1 mM EDTA, pH 8.0.

4° YEME : Difco yeast extract (0.3 %), Difco peptone (0.5 %), oxoid malt extract (0.3 %), glucose (1 %).

Transformation of S. lividans protoplasts

35 1. A culture composed of 25 ml YEME, 34 % sucrose, 0.005 M

MgCl<sub>2</sub>, 0.5 % glycine, in a 250 ml baffled flask, is centrifuged during 30 to 36 hours.

2. The pellet is suspended in 10.3 % sucrose and centrifuged. This washing is repeated once.

3. The mycelium is suspended in 4 ml lysozyme solution (1 mg/ml in P medium with CaCl<sub>2</sub> and MgCl<sub>2</sub> concentrations reduced to 0.0025 M) and incubated at 30°C for 15 to 60 minutes.

4. The solution is mixed by pipetting three times in a 5 ml pipette and incubated for further 15 minutes.

5. P medium (5 ml) is added and mixed by pipetting as in step 4.

6. The solution is filtered through cotton wool and protoplasts are gently sedimented in a bench centrifuge at 800 x G during 7 minutes.

7. Protoplasts are suspended in 4 ml P medium and centrifuged again.

8. Step 7 is repeated and protoplasts are suspended in the drop of P medium left after pouring off the supernatant (for transformation).

9. DNA is added in less than 20 µl TE.

10. 0.5 ml PEG 1 000 solution (2.5 g PEG dissolved in 7.5 ml of 2.5 % sucrose, 0.0014 K<sub>2</sub>SO<sub>4</sub>, 0.1 M CaCl<sub>2</sub>, 0.05 M TRIS-maleic acid, pH 8.0, plus trace elements) is immediately added and pipetted once to mix the components.

11. After 60 seconds, 5 ml of P medium are added and the protoplasts are sedimented by gentle centrifugation.

12. The pellet is suspended in P medium (1 ml).

13. 0.1 ml is plated out on R2YE plates (for transformation dry plates to 85 % of their fresh weight e. g. in a laminar flow cabinet).

14. Incubation at 30°C.

#### A - Construction of a "sfr" gene cassette

A "sfr" gene cassette was constructed to allow subsequent cloning in plant expression vectors.

Isolation of a FokI-BglII fragment from the plasmid pBG39 containing a "sfr" gene fragment led to the loss of the first codons, including the initiation codon, and of the last codons, including the stop codon.

This fragment of the "sfr" gene could be reconstructed in vitro with synthetic oligonucleotides which encode appropriate amino-acids.

The complementary synthetic oligonucleotides were 5'-CATGAGCCCCAGAAC and 3'-TCGGGTCTTGCTGC.

By using such synthetic oligonucleotides, the 5' end of the "sfr" gene could be reformed and the GTG initiation codon substituted for a codon well translated by plant cells, particularly an ATG codon.

The DNA fragment containing the oligonucleotides linked to the "sfr" gene was then inserted into an appropriate plasmid, which contained a determined nucleotide sequence thereafter designated by an "adapter" fragment.

This adapter fragment comprised :

- a TGA termination codon which enabled the last codons of the "sfr" gene to be reformed ;
- appropriate restriction sites which enabled the insertion of the fragment of the nucleotide sequence comprising the "sfr" gene partially reformed with the synthetic oligonucleotides ; this insertion resulted in the reconstruction of an intact "sfr" gene ;
- appropriate restriction sites for the isolation of the entire "sfr" gene.

The "sfr" gene was then inserted into another plasmid, which contained a suitable plant promoter sequence. The plant promoter sequence consisted of the cauliflower mosaic virus promoter sequence (p35S). Of course the invention is not limited to the use of this particular promoter. Other sequences could be chosen as promoters suitable in plants, for example the TR 1'-2' promoter region and the promoter fragment of a Rubisco small

subunit gene from Arabidopsis thaliana hereafter described.

1° Construction of the plasmid pLK56.2 (fig. 3)

5 The construction of plasmid pLK56.2 aimed at obtaining a suitable adaptor including the following sequence of restriction sites : SmaI, BamHI, NcoI, KpnI, BglII, MluI, BamHI, HindIII and XbaI.

10 The starting plasmids used for this construction, pLK56, pJB64 and pLK33 were those disclosed by BOTTERMAN (ref. 11).

The DNA fragments hereafter described were isolated and separated from low melting point agarose (LGA).

15 The plasmid pLK56 was cleaved by the enzymes BamHI and NdeI. A NcoI-NdeI fragment (referred to in the drawings by arc "a" in broken line) obtained from plasmid pJB64 was substituted in pLK56 for the BamHI-NdeI fragment shown at "b". Ligation was possible after filling in the BamHI and NcoI protruding ends with the DNA polymerase I of E. coli (Klenow's fragment).

20 Particularly recircularization took place by means of a T4 DNA ligase. A new plasmid pLK56.3 was obtained.

This plasmid was cleaved by the enzymes XbaI and PstI.

25 The BamHI-PstI fragment of pLK33 (c) (on fig. 3) was substituted for the XbaI-PstI fragment (d) of pLK56.3, after repairing of their respective ends by Klenow's fragment.

30 After recircularization by means of the T4 DNA ligase, the obtained plasmid pLK56.2 contained a nucleotide sequence which comprised the necessary restriction sites for the subsequent insertion of the "sfr" gene.

2° Construction of the plasmid pGSH150 (fig. 4A)

Parallel with the last discussed construction,

there was produced a plasmid containing a promoter sequence recognized by the polymerases of plant cells and including suitable restriction sites, downstream of said promoter sequence in the direction of transcription, which restriction sites are then intended to enable the accommodation of the "sfr" gene then obtainable from pLK56.2, under the control of said plant promoter.

Plasmid pGV825 is described in DEBLAERE et al. (ref. 10). Plasmid pJB63 is from BOTTERMAN (ref. 11).

pGV825 was linearized with PvuII and recircularized by the T4 DNA ligase, resulting in the deletion of an internal PvuII fragment shown at (e), (plasmid pGV956).

pGV956 was then cleaved by BamHI and BglII.

The BamHI-BglII fragment (f) obtained from pJB63 was dephosphorylated with calf intestine phosphatase (CIP) and substituted for the BamHI-BglII fragment of pGV956.

Plasmid pGV1500 was obtained after recircularization by means of T4 DNA ligase.

An EcoRI-HindIII fragment obtained from plasmid pGSH50 was purified. The latter plasmid carried the dual TR 1'-2' promoter fragment described in VELTEN et al., (ref.13). This fragment was inserted in pGV1500, digested with HpaI and HindIII and yielded pGSH150.

This plasmid contains the promoter fragment in front of the 3' end of the T-DNA transcript 7 and a BamHI and ClaI sites for cloning.

### 3' Construction of the plasmid pGSJ260 (fig. 4B)

CP3 is a plasmid derived from pBR322 and which contains the 35S promoter region of cauliflower mosaic virus within a BamHI fragment.

pGSH150 was cut by BamHI and BglII.

The BamHI-BglII fragment (h) of CP3, which contained the nucleotide sequence of p35S promoter, was substituted for the BamHI-BglII fragment (i) in pGSH150 to form plasmid pGSJ250. pGSJ250 was then opened at its BglII

restriction site.

A BamHI fragment obtained from mGV2 (ref. 12) was inserted in pGSJ250 at the BglII site to form plasmid pGSJ260.

However prior to inserting the "sfr" gene obtainable from pLK56.2 into plasmid pGSJ260, it was still desirable to further modify the first in order to permit insertion in a more practical manner. Thus pLK56.2 was further modified as discussed below to yield pGSR1.

Starting from plasmid pGSJ260, two plasmid constructions for subsequent transformations of plant cells were made :

- a first plasmid permitting the expression of the "sfr" gene in the cytoplasm of plant cells, and
- a second plasmid so modified as to achieve transport of the Bialaphos-resistance enzymes to the chloroplasts of plant cells.

First case : plasmid enabling the expression of the "sfr" gene in the cytoplasm of plant cells

Cloning of the sfr gene cassette in a plant expression vector (pGSR2) (fig. 5)

On figure 5A, the nucleotide sequence of the adapter of pLK56.2 is shown. In particular, the locations of BamHI, NcoI, BglII restriction sites are shown.

This adapter fragment was cleaved by the enzymes NcoI and BglII.

Figure 5B shows the FokI-BglII fragment (j) obtained from pBG39. The locations of these two restriction sites are shown on figure 2.

Using synthetic oligonucleotides, the first codons of the "sfr" gene were reformed, particularly the 5' end of the gene in which a ATG initiation codon was substituted for the initial GTG codon.

This FokI-BglII fragment completed with the synthetic oligonucleotides was then substituted in pLK56.2

for the NcoI-BglII fragment of the adapter. The 3' end of the gene was thus reformed too, after recircularization with T4 DNA ligased. The plasmid obtained, pGSR1, thus contained the entire "sfr" gene inserted in its adapter.

The plasmid pGSJ260 was then opened by BamHI (fig. 5C) and the BamHI fragment obtained from pGSR1, which contained the entire "sfr" gene, was inserted into pGSJ260.

The obtained plasmid, pGSR2 (see fig. 6A) contained a pBR322 replicon, a bacterial streptomycin resistance gene (SDM-SP-AD-transferase) and an engineered T-DNA consisting of :

- the border fragments of the T-DNA ;
- a chimeric kanamycin gene which provided a dominant selectable marker in plant cells ; and
- a chimeric "sfr" gene.

The chimeric "sfr" gene consisting of :

- the promoter region of the cauliflower mosaic virus (p35S) ;
- the "sfr" gene cassette as described in fig. 5 ;
- the 3' untranslated region, including the polyadenylation signal of T-DNA transcript 7.

pGSR2 was introduced into Agrobacterium tumefaciens recipient C58ClRif<sup>R</sup> (pGV2260) according to the procedure described by DEBLAERE et al. (ref. 10).

This strain was used to introduce the chimeric "sfr" gene in N. tabacum SR<sub>1</sub> plants.

Two variant plasmids deriving from pGSR2, namely pGSFR280 and pGSFR281, have been constructed. They differ in the untranslated sequence following the transcription initiation site. In pGSR2, this fragment consists of the following sequence :

GAGGACACGCTGAAATCACCAGTCTCGGATCCATG ;

while it consists of :

GAGGACACGCTGAAATCACCAGTCTCTCTACAAATCGATCCATG



in pGSR280 and of

GAGGACACGCTGAAATCACCAGTCTCTCTACAAATCGATG

in pGSFR281, with an ATG codon being the initiation codon of the "sfr" gene. The "sfr" gene is also fused to the TR1'-2' promoter in the plasmid pGSH150 (fig. 4A) yielding pGSFR160 and pGSFR161 (fig. 6B). These plasmids contain slight differences in the pTR2 "sfr" gene configuration : the "sfr" gene is correctly fused to the endogenous gene 2' ATG in pGSFR161 (for sequences see ref. 13), whereas 4 extra base pairs (ATCC) are present just ahead of the ATG codon in pGSFR160. Otherwise, plasmids p65FR161 and p65FR160 are completely identical.

All plasmids are introduced in Agrobacterium by cointegration in the acceptor plasmids pGV2260 yielding the respective plasmids pGSFR1280, pGSFR1281, pGSFR1160 and pGSFR1161.

Second case : construction of a plasmid containing the "sfr" gene downstream of a DNA sequence encoding a transit peptide and suitable for achieving subsequent translocation of the "sfr" gene expression product into plant-cell-chloroplasts

In another set of experiments, the nucleotide sequence which contained the "sfr" gene was fused to a DNA sequence encoding a transit peptide so as to enable its transport into chloroplasts.

A fragment of the "sfr" gene was isolated from the adapter fragment above described and fused to a transit peptide. With synthetic oligonucleotides, the entire "sfr" gene was reconstructed and fused to a transit peptide.

The plasmid (plasmid pATS3 mentioned below) which contained the nucleotide sequence encoding the transit peptide comprised also the promoter sequence thereof.

Construction of the plasmid pGSR4 which contains the "sfr" gene fused to a DNA sequence encoding transit peptide (fig. 7)

Plasmid pLK57 is from BOTTERMAN, (ref.11). Plasmid pATS3 is a pUC19 clone which contains a 2 Kb EcoRI genomic DNA fragment from Arabidopsis thaliana comprising the promoter region and the transit peptide nucleotide sequence of the gene, the expression thereof is the small subunit of ribulose biphosphate carboxylase (ssu). The A. thaliana small subunit was isolated as a 1 500 bp EcoRI-SphI fragment. The SphI cleavage site exactly occurs at the site where the coding region of the mature ssu protein starts.

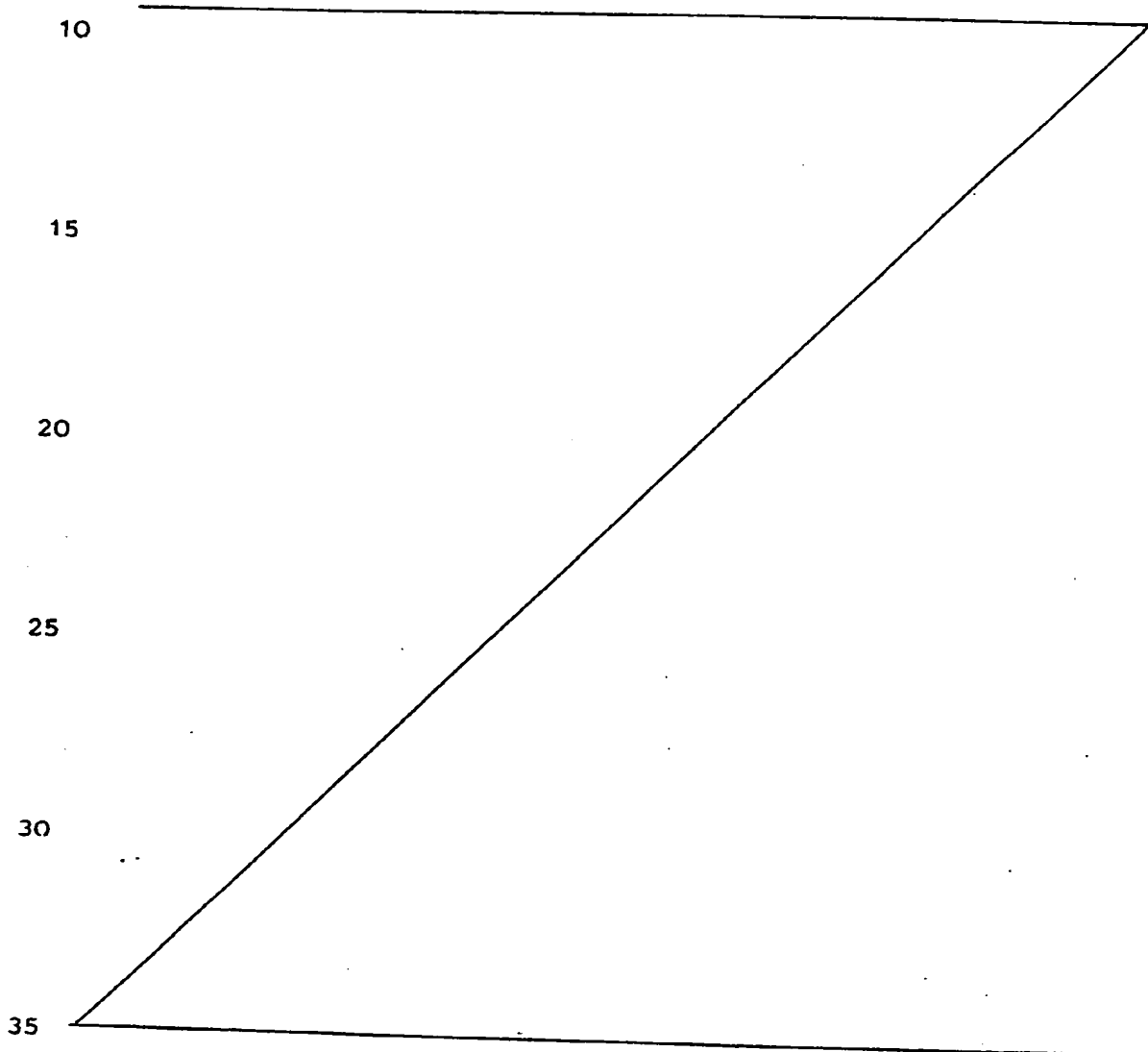
Plasmids pLK57 and pATS3 were opened with EcoRI and SphI. After recircularization by means of the T4 DNA ligase, a recombinant plasmid pLKAB1 containing the sequence encoding the transit peptide (Tp) and its promoter region (Pssu) was obtained.

In order to correctly fuse the "sfr" gene at the cleavage site of the signal peptide, the N-terminal gene sequence was first modified. Since it was observed that N-terminal gene fusions with the "sfr" gene retain their enzymatic activity, the second codon (AGC) was modified to a GAC, yielding an NcoI site overlapping with the ATG initiator site. A new plasmid, pGSSFR2 was obtained. It only differs from pGSR1 (fig. 5B), by that mutation. The NcoI-BamHI fragment obtained from pGSSFR2 was fused at the SphI end of the transit peptide sequence. In parallel, the "sfr" gene fragment was fused correctly to the ATG initiator of the ssu gene (not shown in figures).

#### Introduction of the "sfr" gene into a different plant species

The Bialaphos-resistance induced in plants by the expression of chimeric genes, when the latter have been transformed with appropriate vectors containing said chimeric genes, has been demonstrated as follows. The recombinant plasmids containing the "sfr" gene were introduced separately by mobilization into Agrobacterium strain C58C<sub>1</sub> Rif<sup>R</sup> (pGV2260) according to the procedure described

by DEBLAERE and al., Nucl. Acid. Res., 13, p. 1 477, 1985. Recombinant strains containing hybrid Ti plasmides were formed. These strains were used to infect and transform leaf discs of different plant species, according to a method essentially as described by HORSH and al., 1985, Science, vol. 227. Transformation procedure of these different plant species given by way of example, is described thereafter.



32

1. Leaf disc transformation of Nicotiana tabacum

Used Media are described thereafter :

5	A <sub>1</sub> MS salt/2	+ 1% sucrose 0.8 % agar pH 5.7
10	A <sub>10</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub> 750 mg/l CaCl <sub>2</sub> 2H <sub>2</sub> O 0.5 g/l 2-(N-Morpholino)ethane- sulfonic acid (MES) pH 5.7 30 g/l sucrose
15	A <sub>11</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub> 0.5 g/l MES pH 5.7 2 % glucose 0.8 % agar 40 mg/l adenine
20		+ 1 mg/l 6-Benzylaminopurine (BAP) 0.1 mg/l Indole-3-acetic acid (IAA) 500 mg/l Claforan
25	A <sub>12</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub> 0.5 g/l MES pH 5.7 2 % glucose 0.8 % agar 40 mg/l adenine
30		+ 1 mg/l BAP 200 mg/l claforan
35		

33

A<sub>13</sub> MS-salt/2 + 3 % sucrose  
0.5 MES g/l pH 5.7  
0.7 % agar  
200 mg/l claforan

5

Bacterial medium = min A : (Miller 1972) 60 mM

K<sub>2</sub>HPO<sub>4</sub>, 3H<sub>2</sub>O,  
33 mM KH<sub>2</sub>PO<sub>4</sub> ; 7.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
1.7 M trisodiumcitrat; 1 mM  
MgSO<sub>4</sub> ;  
2 g/l glucose ; 50 mg/l vita-  
mine B1

10

- Plant material :

15

Nicotiana tabacum cv. Petit Havana SR1

Plants are used 6 to 8 weeks after subculture on  
medium A<sub>1</sub>

- Infection :

20

- midribs and edges are removed from leaves.

- Remaining parts are cut into segments of about  
0.25 cm<sup>2</sup> and are placed in the infection medium A<sub>10</sub> (about  
12 segments in a 9 cm Petri dish containing 10 ml A<sub>10</sub>).

- Segments are then infected with 25 µl per Petri  
dish of a late log culture of the Agrobacterium strain  
grown in min A medium.

25

- Petri dish are incubated for 2 to 3 days at low  
light intensity.

- After 2 to 3 days medium is removed and replaced  
by 20 ml of medium A<sub>10</sub> containing 500 mg/l clarofan.

30

- Selection and shoot induction

- The leaf discs are placed on medium A<sub>11</sub> contain-  
ing a selective agent :

100 mg/l kanamycin and

10 to 100 mg/l phosphinotricin.

35

34

- Leaf discs are transferred to fresh medium weekly.

5       - After 3 to 4 weeks regenerating calli arise. They are separated and placed on medium A<sub>12</sub> with the same concentration of selective agent as used for the selection.

- Rooting

10       - After 2 to 3 weeks the calli are covered with shoots, which can be isolated and transferred to rooting medium A<sub>13</sub> (without selection).

- Rooting takes 1 to 2 weeks.

- After a few more weeks, these plants are propagated on medium A<sub>1</sub>.

15       2. Tuber disc infection of Solanum tuberosum (potato)

Used media are described thereafter :

20       C<sub>1</sub> B5-medium       + 250 mg/l NH<sub>4</sub>NO<sub>3</sub>  
                              300 mg/l (CaCH<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>  
                              0.5 g/l MES pH 5.7  
                              0.5 g/l polyvinylpyrrolidone (PVP)  
                              40 g/l mannitol (=0.22M)  
25                            0.8 % agar  
                              40 mg/l adenine

30       C<sub>2</sub> B5-medium       + 250 mg/l NH<sub>4</sub>NO<sub>3</sub>  
                              400 mg/l glutamine  
                              0.5 g/l MES pH 5.7  
                              0.5 g/l PVP  
                              40 g/l mannitol  
                              40 mg/l adenine  
                              0.8 % agar

35

		35	
		+ 0.5 mg/l transzeatine	
		0.1 mg/l IAA	
		500 mg/l clarofan	
5	C <sub>5</sub> MS salt/2	+ 3 % sucrose	
		0.7 % agar	
		pH 5.7	
10	C <sub>7</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub>	
		400 mg/l glutamine	
		0.5 g/l MES pH 5.7	
		0.5 g/l PVP	
		20 g/l mannitol	
15		20 g/l glucose	
		40 mg/l adenine	
		0.6 % agarose	
		+ 0.5 mg/l transzeatine	
20		0.1 mg/l IAA	
		500 mg/l clarofan	
	C <sub>8</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub>	
		400 mg/l glutamine	
25		0.5 g/l MES pH 5.7	
		0.5 g/l PVP	
		20 g/l mannitol	
		20 g/l glucose	
		40 mg/l adenine	
30		0.6 % agarose	
		+ 200 mg/l clarofan	
		1 mg/l transzeatine	

35

36

C<sub>9</sub> B5-medium

+ 250 mg/l NH<sub>4</sub>NO<sub>3</sub>  
 400 mg/l glutamine  
 0.5 g/l MES pH 5.7  
 0.5 g/l PVP  
 20 g/l mannitol  
 20 g/l glucose  
 40 mg/l adenine  
 0.6 % agarose

5

10

+ 1 mg/l transzeatine  
 0.01 mg/l Gibberellic acid A<sub>3</sub>  
 (GA<sub>3</sub>)  
 100 mg/l clarofan

15

C<sub>11</sub> MS salt/2

+ 6 % sucrose  
 0.7 % agar

20

Bacterial medium = min A : (Miller 1972 60 mM K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O;  
 33 mM KH<sub>2</sub>PO<sub>4</sub>; 7.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>;  
 1.7 trisodiumcitrat; 1 mM  
 MgSO<sub>4</sub> ;  
 2 g/l glucose; 50 mg/l vitami-  
 ne B1.

25

- Plant material

Tubers of Solanum tuberosum c.v Berolina  
 c.v Désirée

- Infection

30

- Potatoes are peeled and washed with water.  
 - Then they are washed with concentrated commer-  
 cial bleach for 20 minutes, and  
 - rinsed 3 to 5 times with sterile water.  
 - The outer layer is removed (1 to 1.5 cm)  
 - The central part is cut into discs of about 1  
 cm<sup>2</sup> and 2 to 3 mm thick.

35

- Discs are placed on medium C<sub>1</sub> (4 pieces in a 9



cm Petri dish).

- 10  $\mu$ l of a late log culture of an Agrobacterium strain grown in min A medium is applied on each disc.

5       - Discs are incubated for 2 days at low light intensity.

- Selection and shoot induction

- Discs are dried on a filter paper and transferred to medium C<sub>2</sub> with 100 mg/l kanamycin.

10       - After one month small calli are removed from the discs and transferred to medium C<sub>7</sub> containing 50 mg/l kanamycin.

- After a few more weeks, the calli are transferred to medium C<sub>8</sub> containing 50 mg/l kanamycin.

15       - If little shoots start to develop, the calli are transferred to elongation medium C<sub>9</sub> containing 50 mg/l Kanamycin.

- Rooting

20       - Elongated shoots are separated and transferred to rooting medium C<sub>11</sub>.

- Rooted shoots are propagated on medium C<sub>5</sub>.

3. Leaf disc infection of Lycopersicum esculentum (tomato)

Used media are described thereafter

25       A<sub>1</sub> MS salt/2       + 1 % sucrose  
                                  0.8 % agar  
                                  pH 5.7

30       B<sub>1</sub> B5-medium       + 250 mg/l NH<sub>4</sub>NO<sub>3</sub>  
                                  0.5 g/l MES pH 5.7  
                                  0.5 g/l PVP  
                                  300 mg/l Ca (H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>  
                                  2 % glucose  
                                  40 mg/l adenine  
                                  40 g/l mannitol

35

		38	
	B <sub>2</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub>	
		0.5 g/l MES pH 5.7	
		0.5 g/l PVP	
5		400 mg/l glutamine	
		2 % glucose	
		0.6 % agarose	
		40 mg/l adenine	
		40 g/l mannitol	
10		+ 0.5 mg/l transzeatine	
		0.01 mg/l IAA	
		500 mg/l claforan	
15	B <sub>3</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub>	
		0.5 g/l MES pH 5.7	
		0.5 g/l PVP	
		400 mg/l glutamine	
		2 % glucose	
20		0.6 % agarose	
		40 mg/l adenine	
		30 g/l mannitol	
		+ 0.5 mg/l transzeatine	
25		0.01 mg/l IAA	
		500 mg/l clarofan	
	B <sub>4</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub>	
		0.5 g/l MES pH 5.7	
30		0.5 g/l PVP	
		400 mg/l glutamine	
		2 % glucose	
		0.6 % agarose	
		40 mg/l adenine	
35		20 g/l mannitol	

		39	
		+ 0.5 mg/l transzeatine	
		0.01 mg/l IAA	
		500 mg/l clarofan	
5	B <sub>5</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub>	
		0.5 g/l MES pH 5.7	
		0.5 g/l PVP	
		400 mg/l glutamine	
10		2 % glucose	
		0.6 % agarose	
		40 mg/l adenine	
		10 g/l mannitol	
15		+ 0.5 mg/l transzeatine	
		0.01 mg/l IAA	
		500 mg/l clarofan	
20	B <sub>6</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub>	
		0.5 g/l MES pH 5.7	
		0.5 g/l PVP	
		400 mg/l glutamine	
		2 % glucose	
		0.6 % agarose	
25		40 mg/l adenine	
		+ 0.5 mg/l transzeatine	
		0.01 mg/l IAA	
		200 mg/l clarofan	
30	B <sub>7</sub> B5-medium	+ 250 mg/l NH <sub>4</sub> NO <sub>3</sub>	
		0.5 g/l MES pH 5.7	
		0.5 g/l PVP	
		400 mg/l glutamine	
35		2 % glucose	
		0.6 % agarose	

- 40  
40 mg/l adenine
- + 1 mg/l transzeatine  
200 mg/l clarofan
- 5  
B<sub>8</sub> MS salt/2 + 2 % sucrose  
0.5 g/l MES pH 5.7  
0.7 % agar
- 10  
B<sub>9</sub> B5-medium + 250 mg/l NH<sub>4</sub>NO<sub>3</sub>  
0.5 g/l MES pH 5.7  
0.5 g/l PVP  
2 % glucose  
0.6 % agarose  
40 mg/l adenine
- 15  
+ 1 mg/l transzeatine  
0.01 mg/l GA<sub>3</sub>
- 20  
Bacterial medium = min A : (Miller 1972) 60 mM  
K<sub>2</sub>HPO<sub>4</sub> · 3H<sub>2</sub>O ;  
33 mM KH<sub>2</sub>PO<sub>4</sub>; 7.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>;  
1.7 M trisodiumcitrat; 1 mM  
MgSO<sub>4</sub> ;
- 25  
2 g/l glucose; 50 mg/l vitami-  
ne B1

- Plant material

Lycopersicum esculentum cv. Lucullus.

Plants are used 6 weeks after subculture on medium A<sub>1</sub>.

30 - Infection

- Midrib is removed from the leaves.
- Leaves are cut in segments of about 0.25 to 1  
cm<sup>2</sup> (the edges of the leaves are not wounded, so that only  
maximum 3 sides of the leaf pieces is wounded).
- 35 - Segments are placed in infection medium B<sub>1</sub>

(upside down), about 10 segments in a 9 cm Petri dish.

5       - Segments are then infected with 20  $\mu$ l per Petri dish of a late log culture of the Agrobacterium strain grown in min A medium.

      - Petri dishes incubate for 2 days at low light intensity.

      - Medium is removed after 2 days and replaced by 20 ml of medium B<sub>1</sub> containing 500 mg/l clarofan.

10      - Selection and shoot induction

      - The leaf discs are placed in medium B<sub>2</sub> + 50 or 100 mg/l kanamycin.

      - Each 5 days the osmotic pressure of the medium is lowered by decreasing the mannitol concentration, transfers are done consecutively in medium B<sub>3</sub>, B<sub>4</sub>, B<sub>5</sub>,  
15      and B<sub>6</sub>.

      - After one month calli with meristems are separated from the leaf discs and placed on medium B<sub>7</sub> with 50 or 100 mg/l kanamycin.

20      - Once little shoots have formed, calli are transferred to elongation medium B<sub>9</sub> with 50 or 100 mg/l kanamycin.

      - Rooting

25      - Elongated shoots are separated and transferred to medium B<sub>8</sub> for rooting.

      - Plants are propagated on medium A<sub>1</sub>.

#### Greenhouse tests for herbicide resistance

##### Material and method

30      In this experiment, two herbicides comprising phosphinotricin as active ingredient, are used.

      These compounds are those commercially available under the registered trademarks BASTA<sup>R</sup> and MEIJI HERBIACE<sup>R</sup>.

35      These products are diluted to 2 % with tap water. Spraying is carried out on a square metre area from the

four corners. Temperature of the greenhouse is about 22°C for tobaccos and tomato, and above 10°C to 15°C for potato.

5

### Results

#### - Tobacco spraytest

a) Nicotiana tabacum cv. Petit Havana SR1 plants transformed with the chimeric "sfr" genes as present in pGSFR1161 or pGSFR1281, as well as untransformed control plants (from 10 cm to 50 cm high) are treated with 20 l BASTA<sup>R</sup>/ha. Control SR1 plants die after 6 days, while transformed plants are fully resistant to 20 l BASTA<sup>R</sup>/ha and continue growing undistinguishable from untreated plants. No visible damage is detected, also the treatment is repeated every two weeks. The treatment has no effect in subsequent flowering. The recommended dose of BASTA<sup>R</sup> herbicide in agriculture is 2.5-7.5 l/ha.

b) A similar experiment is performed using 8 l/ha MEIJI HERBIACE<sup>R</sup>. The transformed plants (the same as above) are fully resistant and continue growing undistinguishable from untreated plants. No visible damage is detectable.

#### - Potato spraytest

Untransformed and transformed potato plants (Solanum tuberosum cv. Berolina) (20 cm high) with the chimeric "sfr" gene as present in pGSFR1161 or pGSFR1281 are treated with 20 l BASTA<sup>R</sup>/ha. Control plants die after 6 days while the transformed plants do not show any visible damage. They grow undistinguishable from untreated plants.

#### - tomato spraytest

Untransformed and transformed tomato plants (lycopersium esculentum c.v. luculus) (25 cm high) with the chimeric "sfr" gene as present in pGSFR1161 and pGSFR1281 are treated with 20 l BASTA<sup>R</sup>/ha. control plants

die after six days while transformed plants are fully resistant. They do not show any visible damage and grow undistiguishable from untreated plants.

- 5           - Growth control of phytopathogenic fungi with transformed plants

          In another set of experiments, potato plants expressing chimeric "sfr" genes as present in pGSFR1161 or pGSFR1281 are grown in a greenhouse compartment at 20°C under high humidity. Plants are innoculated by spraying 10   1 ml of a suspension of  $10^6$  Phytophthora infestans spores per ml. Plants grow in growth chambers (20°C, 95 % humidity, 4 000 lux) until fungal disease symptoms are visible (one week). One set of the plants are at that moment sprayed with Bialaphos at a dose of 8 l/ha. Two weeks later, 15   untreated plants are completely ingested by the fungus. The growth of the fungus is stopped on the Bialaphos treated plants and no further disease symptoms evolve. The plants are effectively protected by the fungicide action of Bialaphos.

- 20           - Transmission of the PPT resistance through seeds  
          Transformed tobacco plants expressing the chimeric "sfr" gene present in pGSFR1161 and pGSFR1281 are brought to flowering in the greenhouse. They show a normal fertility.

- 25           - About 500 F1 seeds of each plant are sown in soil, F1 designating seeds of the first generation, i.e directly issued from the originally transformed plants. When seedlings are 2-3 cm high, they are sprayed with 8 l BASTA<sup>R</sup>/ha. 7 days later, healthy and damaged plants can be 30   distinguished in a ratio of approximately 3 to 1. this shows that PPT resistance is inherited as a dominant marker encoded by a single locus.

- 10 resistant F1 seedlings are grown to maturity and seeds are harvested. F2 seedlings are grown as 35   described above and tested for PPT-resistance by spraying

BASTA<sup>R</sup> at a dose of 8 l/ha. Some of the F1 plants produce F2 seedlings which are all PPT-resistant showing that these plants are homozygous for the resistance gene. The invention also concerns plant cells and plants non-essentially-biologically-transformed with a GS inhibitor-inactivating-gene according to the invention.

In a preferred embodiment of the invention, plant cells and plants are non-biologically-transformed with the "sfr" gene hereabove described.

Such plant cells and plants possess, stably integrated in their genome, a non-variety-specific character which render them able to produce detectable amounts of phosphinotricin-acetyl transferase.

This character confers to the transformed plant cells and plants a non-variety-specific enzymatic activity capable of inactivating or neutralizing GS inhibitors like Bialaphos and PPT.

Accordingly, plant cells and plants transformed according to the invention are rendered resistant against the herbicidal effects of Bialaphos and related compounds.

Since Bialaphos was first described as a fungicide, transformed plants can also be protected against fungal diseases by spraying with the compound several times.

In a preferred embodiment, Bialaphos or related compounds is applied several times, particularly at time intervals of about 20 to 100 days.

The invention also concerns a new process for selectively protecting a plant species against fungal diseases and selectively destroying weeds in a field comprising the steps of treating a field with an herbicide, wherein the plant species contain in their genome a DNA fragment encoding a protein having an enzymatic activity capable of neutralizing or inactivating GS inhibitors and wherein the used herbicide comprises as



active ingredient a GS inhibitor.

5 It comes without saying that the process according to the invention can be employed with the same efficiency, either to only destroy weeds in a field, if plants are not infected with fungi, either to only stop the development of fungi if the latter appears after destruction of weeds.

10 In a preferred embodiment of the process according to the invention, plant species are transformed with a DNA fragment comprising the "sfr" gene as described hereabove, and the used herbicide is PPT or a related compound.

15 Accordingly, a solution of PPT or related compound is applied over the field, for example by spraying, several times after emergence of the plant species to be cultivated, until early and late germinating weeds are destroyed.

It is quite evident that before emergence of plant species to be cultivated, the field can be treated with an herbicidal composition to destroy weeds.

20 On the same hand, fields can be treated even before the plant species to be cultivated are sowed.

Before emergence of the desired plant species, fields can be treated with any available herbicide, including Bialaphos-type herbicides.

25 After emergence of the desired plant species, Bialaphos or related compound is applied several times.

In a preferred embodiment, the herbicide is applied at time intervals of about from 20 to 100 days.

30 Since plants to be cultivated are transformed in such a way as to resist to the herbicidal effects of Bialaphos-type herbicides, fields can be treated even after emergence of the cultivated plants.

35 This is particularly useful to totally destroy early and late germinating weeds, without any effect on the plants to be produced.

Preferably, Bialaphos or related compound is

applied at a dose ranging from about 0.4 to about 1.6 kg/ha, and diluted in a liquid carrier at a concentration such as to enable its application to the field at a rate ranging from about 2 to about 8 l/ha.

5 There follows examples, given by way of illustration, of some embodiments of the process with different plant species.

- Sugarbeets

10 The North European sugarbeet is planted from March 15 up to April 15, depending upon the weather condition and more precisely on the precipitation and average temperature. the weeds problems are more or less the same in each country and can cause difficulties until the crop closes its canopy around mid-July.

15 Weed problems can be separated in three situations :

- early germination of the grassy weeds,
- early germinating broadleaved weeds,
- 20 - late germinating broadleaved weeds.

Up to now, pre-emergence herbicides have been successfully used. Such compounds are for example those commercially available under the registered trademarks : PYRAMIN<sup>R</sup>, GOLTIX<sup>R</sup> and VENZAR<sup>R</sup>. However, the susceptibility to dry weather conditions of these products as well as the lack of residual activity to control late germinating weeds have led the farmer to use post-emergence products in addition to pre-emergence ones.

25 Table (I) thereafter indicates the active ingredients contained in the herbicidal compositions cited in the following examples.

35



TABLE (I)

	Commercial Name	Active Ingredient	Formulation
5	AVADEX <sup>R</sup>	Diallate	EC 400 g/l
	AVADEX BW <sup>R</sup>	Triallate	EC 400 g/l
	GOLTIX <sup>R</sup>	Metamitron	WP 70 %
	RONEET <sup>R</sup>	Cycloate	EC 718 g/l
	TRAMAT <sup>R</sup>	Ethofumerate	EC 200 g/l
10	FERVINAL <sup>R</sup>	Alloxydime-sodium	SP 75 %
	BASTA <sup>R</sup>	Phosphinotricin	200 g/l
	PYRAMIN FL <sup>R</sup>	Chloridazon	SC 430 g/l

15 According to the invention, post-emergence herbicides consist of Bialaphos or related compounds, which offer a good level of growth control of annual grasses (Bromus, Avena spp., Alopecurus, POA) and broadleaves (Galium, Polygonum, Senecio, Solanum, Mercurialis).

20 Post-emergence herbicides can be applied at different moments of the growth of sugarbeet ; at a cotyledon level, two-leave level or at a four-leave level.

25 Table (II) thereafter represents possible systems of field-treatment, given by way of example.

In those examples, the post-emergence herbicide of the class of Bialaphos used is BASTA<sup>R</sup>, in combination with different pre-emergence herbicides. Concentrations are indicated in l/ha or kg/ha.

30

35

TABLE (11)

POSSIBLE WEEDCONTROL SYSTEMS IN SUGARBEETS, BASED ON THE USE OF BASTA<sup>R</sup>, PROVIDING BEETS ARE MADE RESISTANT AGAINST THE LATTER CHEMICAL (in lt or kg/ha).

	Pre-sowing	Pre-emergence	Cotyledons	Two-leaves	Four leaves
1.	AVADEX <sup>R</sup> 3.5 lt	-	BASTA <sup>R</sup> 3 lt	BASTA <sup>R</sup> /Lramat 3 lt 1.5 lt	-
2.	AVADEX <sup>R</sup> 3.5 lt	GOLTIX <sup>R</sup> 4 kg	-	-	-
3.	RONEET <sup>R</sup> 4 lt	GOLTIX <sup>R</sup> 5 kg	-	-	-
4.	RONEET <sup>R</sup> 4 lt	GOLTIX <sup>R</sup> 2.5 kg	-	BASTA <sup>R</sup> 3 lt	-
5.	TRAMAT <sup>R</sup> 5 lt	-	-	BASTA <sup>R</sup> 3 lt	BASTA <sup>R</sup> /GOLTIX <sup>R</sup> 2 lt 2 kg
6.	-	GOLTIX <sup>R</sup> 2.5 kg	-	BASTA <sup>R</sup> 3 lt	-
7.	-	-	BASTA <sup>R</sup> /Lramat 3 lt 1.7 lt	-	BASTA <sup>R</sup> /GOLTIX <sup>R</sup> 3 lt 2 kg
8.	PYRAMIN <sup>R</sup> 6 lt	-	BASTA <sup>R</sup> 3 lt	Venzar 1 kg	-
9.	-	-	BASTA <sup>R</sup> 3 lt	BASTA <sup>R</sup> /GOLTIX <sup>R</sup> 3 lt 2 kg	-
10.	DIALATE <sup>R</sup> 3.5 lt	PYRAMIN <sup>R</sup> 6 lt	-	BASTA <sup>R</sup> /Metamitron 3 lt 1 kg	-

- Potatoes

Potatoes are grown in Europe on about  $8.10^6$  Ha. The major products used for weed control are Linuron/monolinuron or the compound commercially available under the denomination METRABUZIN

These products perform well against most weedspecies.

However, weeds such as Galium and Solanum plus late germinating Chenopodium and Polygonum are not always effectively controlled, while control of the annual grasses is also sometime erratic.

Once again, late germinating broadleaved weeds are only controllable by post-emergence applications of herbicides such as BASTA<sup>R</sup>.

Table (III) thereafter represents some examples given by way of example of field-treatment in the case of potatoes.

TABLE (III)

Weeds control systems in potatoes, based on the use of BASTA<sup>R</sup>, providing potatoes are rendered resistant to BASTA<sup>R</sup>.

Linuron + monolinuron (375 g + 375 g/ha) prior to emergence	
BASTA <sup>R</sup>	3-4 lt/ha after emergence (5-15 cm)
BASTA <sup>R</sup> /fluazifop-butyl	3-4 lt/ha + 2 lt/ha after emergence (5-15 cm)

Linuron	WP 50 % (AFALON <sup>R</sup> )
Monolinuron	WP 47.5 % (ARESSIN <sup>R</sup> )
fluazifop-butyl	EL 250 g/l (FUSILADE <sup>R</sup> )

The strains pGSJ260 and pBG39 used hereabove have

been deposited on December 12nd, 1985, at the "German Collection of Micro-organisms" (DEUTSCHE SAMMLUNG VON MIKROORGANISMEN) at Göttingen, Germany. They received the deposition numbers DSM 3 606 and DSM 3 607 respectively.

5 Further embodiments of the invention are described hereafter with reference to the figures in which :

- fig. 8 shows the restriction map of a plasmid pJS1 containing another Bialaphos-resistance-gene ;

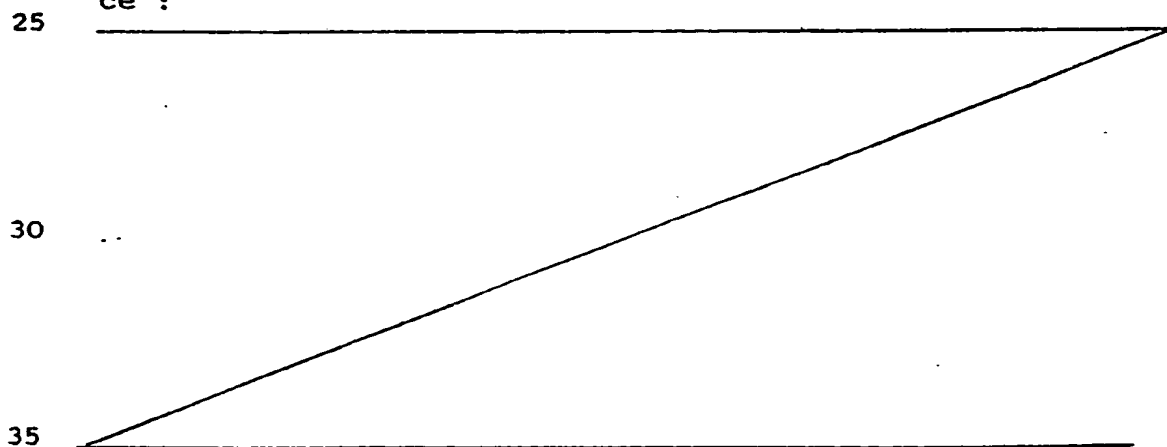
10 - fig. 9 shows the nucleotide sequence of the "sfrsv" gene containing the resistance gene ;

- fig. 10 shows the amino acid homology of "sfrsv" gene and "sfr" gene,

15 - fig. 11 shows the construction of a plasmid, given by way of example, which contains the "sfrsv" gene and suitable for the transformation of plant cells.

Another Bialaphos-resistance-gene has been isolated from another Bialaphos-producing-strains, i.e. streptomyces viridochromogenes. This second resistance-gene is thereafter designated by "sfrsv" gene.

20 This second preferred DNA fragment according to the invention, for the subsequent transformation of plant cells, consists of a nucleotide sequence coding for at least part of a polypeptide having the following sequence :



51

V S P E R R P V E I R P A T A A D M  
5 A A V C D I V N H Y I E T S T V N F  
R T E P Q T P Q E W I D D L E R L Q  
10 D R Y P W L V A E V E G V V A G I A  
Y A G P W K A R N A Y D W T V E S T  
15 V Y V S H R H Q R L G L G S T L Y T  
H L L K S M E A Q G F K S V V A V I  
20 G L P N D P S V R L H E A L G Y T A  
R G T L R A A G Y K H G G W H D V G  
25 F W Q R D F E L P A P P R P V R P V  
T Q I \*

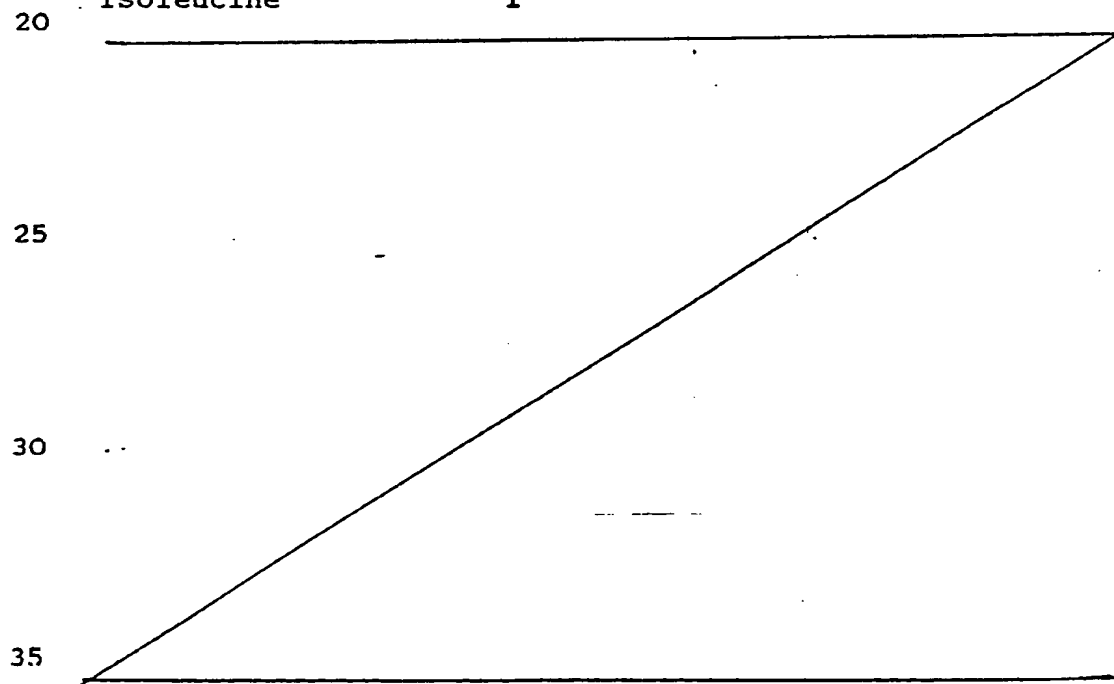
30

35

which part of said polypeptide is of sufficient length to confer protection against Bialaphos-"plant-protecting-capability" to plant cells, when incorporated genetically and expressed therein. Reference will also be made here-  
5 after to the "plant-protecting-capability" against Bialaphos of the abovesaid nucleotide sequence.

Meaning of the designation of amino acids by a single letter is given thereafter.

10	Alanine	A	Leucine	L
	Arginine	R	Lysine	K
	Asparagine	N	Methionine	M
	Aspartic Acid	D	Phenylalanine	F
	Cysteine	C	Proline	P
15	Cystine	C	Serine	S
	Glycine	G	Threonine	T
	Glutamic Acid	E	Tryptophan	W
	Glutamine	Q	Tyrosine	Y
	Histidine	H	Valine	V
20	Isoleucine	I		







or of a part thereof expressing a polypeptide having plant-protecting capability against Bialaphos ;

There follows hereafter the description of experiments carried out for the isolation of the "sfrsv" resistance gene, the construction of expression vectors which contain the resistance gene and which allow the subsequent transformation of plant cells, in order to render them resistant to GS inhibitors.

10      Cloning of the bialaphos-resistance-"sfrsv" gene from Streptomyces viridochromogenes

The strain Streptomyces viridochromogenes DSM

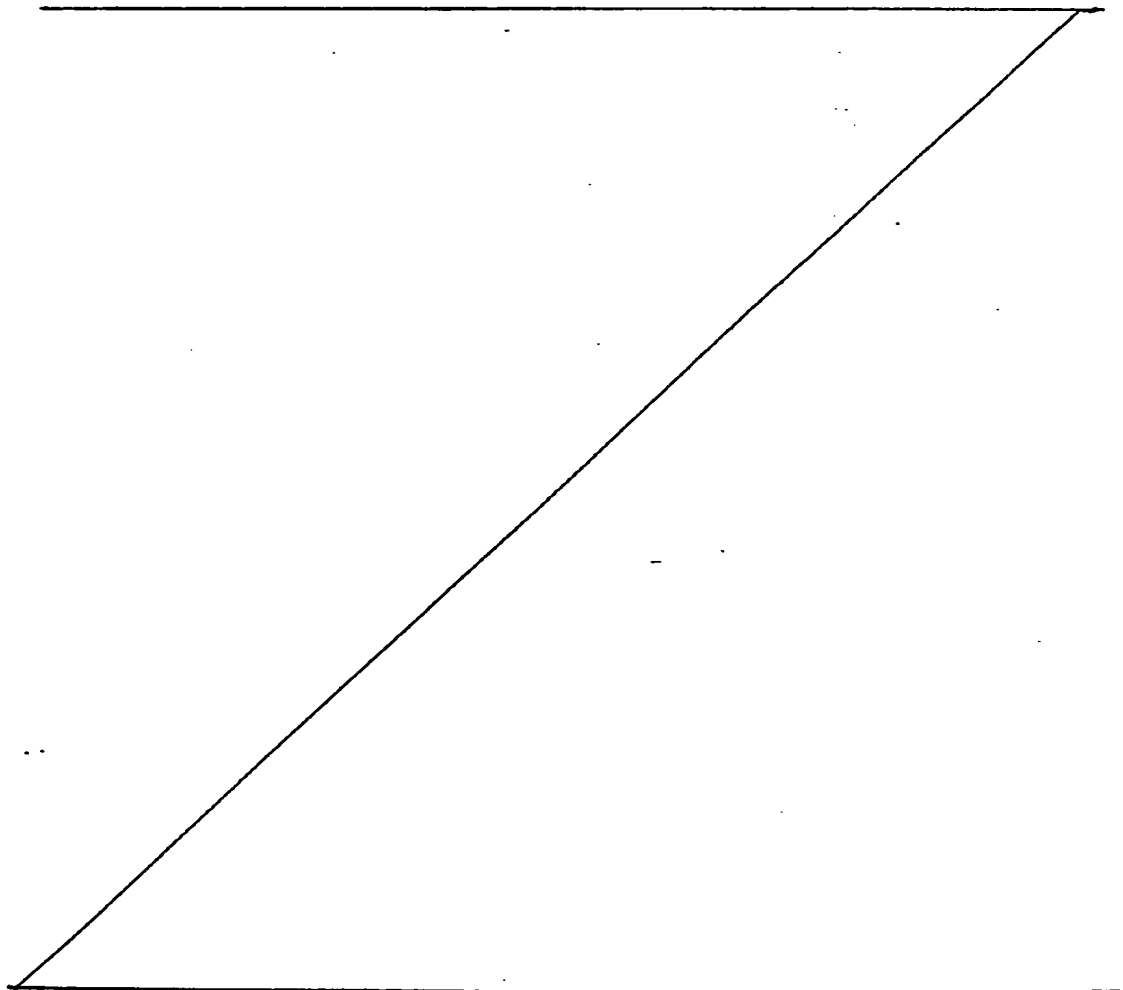
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40736 (ref 1) was grown and total DNA of this strain was prepared according to standard techniques. DNA samples were digested respectively with PstI, SmaI and Sau3AI in three different reactions and separated on an agarose gel, together with plasmid DNA from pGSR1 (fig. 5B) digested with BamHI. In a Southern analysis the DNA was blotted on a nitrocellulose filter and hybridized with the labeled BamHI fragment from pGSR1 containing the "sfr" gene. In all four lanes of the gel, a restriction fragment was showing strong homology with the probe : a PstI fragment of about 3 kb, a SmaI fragment of about 1.2 kb and Sau3AI fragment of 0.5 kb. In order to clone this gene, PstI restriction fragments were directly cloned in the Escherichia coli vector pUC8. 3000 colonies obtained after transformation were transferred to nitrocellulose filters, and hybridized with the "sfr" probe. Positive candidates were further tested for their growth on minimal medium plates containing 300 µg/ml PPT. One transformant that grew on PPT-containing-medium was further analysed. The plasmid map and relevant restriction sites of this plasmid pJS1 are represented in fig. 8. The strain MC1061 (pJS1) has been deposited on March 06, 1987 at the DEUTSCHE SAMMLUNG VON MIKROORGANISMEN (DSM) under deposition number DSM 4023. The clone restriction fragment has been sequenced according to the Maxam and Gilbert method and the coding region of the gene could be identified through homology. The sequence of the "sfrsv" gene is represented in fig.9 and the homology on the nucleotide and amino acid sequence level with "sfr" gene is shown in fig. 10.

#### Expression of the "sfrsv" gene

A "sfrsv gene cassette" was also constructed to allow subsequent cloning in plant expression vectors. A BanII-BglII fragment containing the "sfrsv" coding region without the initiation codon GTG was isolated from pJS1.

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This fragment was ligated in the vector pLK56-2 digested with NcoI and BglII, together with a synthetic oligonucleotide 5'-CATGAGCC-3', similar with the one described for "sfr" gene and shown in fig. 5. The construction of pGSR1SV is schematically shown in fig. 11. Since similar cassettes of both genes are present in respectively pGSR1 and pGSR1SV, previous described constructions for the expression of the "sfr" gene in plants can be repeated.

Enzymatic analysis of crude extracts from E. coli strains carrying plasmid pGSR1SV demonstrated the synthesis of an acetylase which could acetylate PPT. This was shown by thin layer chromatography of the reaction products.

The "sfrsv" gene was then inserted into the plasmid vector pGSJ260 (fig. 4B) under the control of the CaMV 35s promoter, to yield a plasmid pGS2SV, similar to pGSR2 (fig. 6A) except that the "sfrsv" gene is substituted for the "sfr" gene.

It is clear that herbicide resistance gene of the above type may be obtained from many other microorganism that produce PPT or PPT derivatives. Herbicide resistance gene can then be incorporated in plant cells with a view of protecting them against the action of such Glutamine Synthetase inhibitors. For instance, a Bialaphos-resistance-gene is obtained from Kitasatosporia (ref. 15).

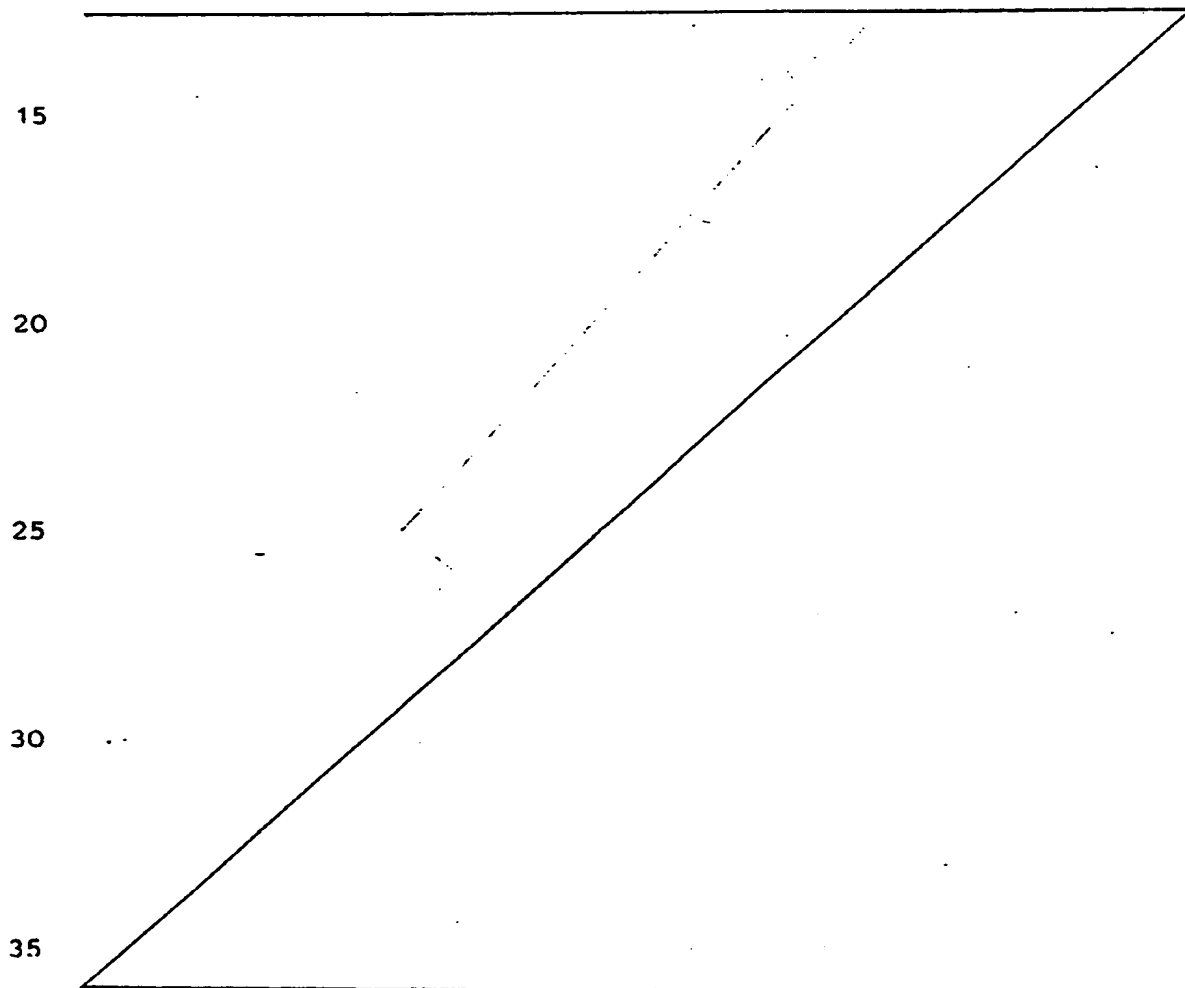
Transformed plant cells and plants which contain the "sfrsv" resistance gene can be obtained with plasmid pGSR2SV, using the same Agrobacterium-mediated-transformation system as hereabove described for the transformation of different plant species with the "sfr" gene.

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Plants are regenerated and tested for their resistance with similar spraying tests as described hereabove. All plants behaved similarly and show resistance against herbicides consisting of glutamine synthetase inhibitors.

Finally, the invention also pertains to the combination of the plants resistant to an inhibitor of glutamine synthetase as defined above with the corresponding inhibitor of glutamine synthetase for use in the production of the cultures of said plants free from weeds.



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1986

## CLAIMS

1. Process for controlling the action in plant cells and plants of a glutamine synthetase inhibitor when  
5 contacted herewith, which comprises providing said plants with a heterologous DNA including a foreign nucleotide sequence, capable of being expressed in the form of a protein in said plant cells and plants, under conditions such as to cause said heterologous DNA to be integrated  
10 stably through generations in the cells of said plants, and wherein said protein has an enzymatic activity capable of causing inactivation or neutralization of said glutamine synthetase inhibitor.

2. Process according to claim 1, wherein the  
15 heterologous DNA fragment comprises a foreign nucleotide sequence coding for a polypeptide having a PPT acetyl transferase activity.

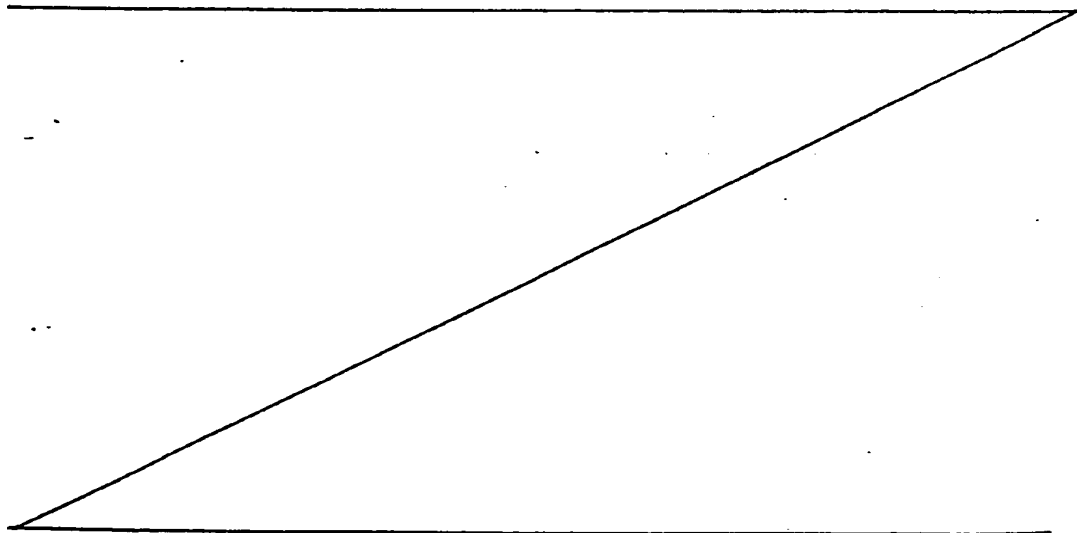
3. Process according to the claims 1 to 2, wherein the heterologous DNA fragment belongs to the genome of a  
20 Streptomyces.

4. Process according to any of the claims 1 to 3, wherein the heterologous DNA fragment comprises a foreign

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nucleotide sequence coding for the following amino acid  
sequence :

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238

1:0

318

363

19

408

453

20.

543

568

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633

**678**

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723



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in which X represents MET or VAL, or a part of a polypeptide of sufficient length to possess said enzymatic activity and, when said DNA fragment is expressed in plant cells, to protect the latter against the herbicidal activity of glutamine synthetase inhibitors.

5. Process according to any of the claims 1 to 4, wherein the heterologous DNA fragment comprises the following nucleotide sequence :

```

10      123      GTG AGC CCA GAA
      CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
      228
      GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
      273
15      AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
      318
      CTC GTC CGT CTG CCG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
      363
20      GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
      408
      CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
      453
      CCC CGC CAC CAG CCG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
      498
25      CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
      543
      GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
      588
      CTC GGA TAT GCC CCC CGC GGC ATG CTG CCG GCG GCC GGC TTC AAG
      633
30      CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
      678
      CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
      723
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or a part thereof expressing a polypeptide capable of inactivating said glutamine synthetase inhibitor.

5 6. Process according to any of claims 1 to 5, wherein the initiation codon ATG is substituted for the initiation codon GTG of the sequence defined in claim 5.

7. Process according to any of claims 1 to 3, wherein the heterologous DNA fragment comprises a foreign nucleotide sequence coding for the following amino acid sequence :

10 V S P E R R P V E I R P A T A A D M

A A V C D I V N H Y I E T S T V N F

15 R T E P Q T P Q E W I D D L E R L Q

D R Y P W L V A E V E G V V A G I A

20 Y A G P W K A R N A Y D W T V E S T

V Y V S H R H Q R L G L G S T L Y T

25 H L L K S M E A Q G F K S V V A V I

G L P N D P S V R L H E A L G Y T A

30 R G T L R A A G Y K H G G W H D V G

F W Q R D F E L P A P P R P V R P V

35 T Q I \*

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or a part of a polypeptide of sufficient length to possess said enzymatic activity and, when said DNA fragment is expressed in plant cells, to protect the latter against the herbicidal activity of glutamine synthetase inhibitors.

8. Process according to claim 7, wherein the heterologous DNA fragment comprises the following nucleotide sequence :

10 TAAAGAGGTGCCCGCCACCCGCTTTCGCAGAACACCGAAGGAGACCACAC  
↓  
GTGAGCCCAGAACGACGCCCGGTGAGATCCGTCCCGCCACCGCCGCCGA  
CATGGCGGCGGTCTGCGACATCGTCAATCACTACATCGAGACGAGCACGG  
15 TCAACTTCCGTACGGAGCCGCAGACTCCGCAGGAGTGGATCGACGACCTG  
GAGCGCCTCCAGGACCGCTACCCCTGGCTCGTCGCCGAGGTGGAGGGCGT  
CGTCGCCGGCATCGCCTACGCCGGCCCCCTGGAAGGCCCGCAACGCCTACG  
20 ACTGGACCGTCGAGTCGACGGTGTACGTCTCCACCCGGCACCAGCGGCTC  
GGACTGGGCTCCACCCTCTACACCCACCTGCTGAAGTCCATGGAGGCCCA  
GGGCTTCAAGAGCGTGGTCCGCGTCATCGGACTGCCCAACGACCCGAGCG  
25 TGCGCCTGCACGAGGCGCTCGGATACACCGCGCGGGACGCTGCGGGCA  
GCCGGCTACAAGCACGGGGGCTGGCACGACGTGGGGTTCTGGCAGCGCGA  
30 CTTGAGCTGCCGGCCCCGCCCCGCCCCGTCCGGCCCGTCACACAGATCT  
GAGCGGAGAGCGCATGGC

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or a part thereof expressing a polypeptide capable of inactivating said glutamine synthetase inhibitor.

5           9. In a process for producing plants and reproduction material of said plants including a heterologous genetic material stably integrated therein and capable of being expressed in said plants or reproduction material in the form of a protein capable of inactivating or neutralizing the activity of a glutamine  
10       synthetase inhibitor, comprising the non biological steps of producing plants cells or plant tissue including said heterologous genetic material from starting plant cells or plant tissue not able to express that inhibiting or neutralizing activity, regenerating plants or reproduction  
15       material of said plants or both from said plant cells or plant tissue including said genetic material and, optionally, biologically replicating said last mentioned plants or reproduction material or both, wherein said steps of producing said plant cells or plant tissue  
20       including said heterologous genetic material comprises transforming said starting plant cells or plant tissue with a DNA recombinant containing a nucleotide sequence encoding said protein as well as the regulatory elements selected among those which are capable of enabling the  
25       expression of said nucleotide sequence in said plant cells or plant tissue and to cause the stable integration of said nucleotide sequence in said plant cells and tissue, as well as in the plant and reproduction material processed therefrom throughout generations.

30           10. The process according to claim 9, wherein starting cells are transformed with a recombinant DNA which contains the fragment of any one of the claims 1 to 8 recombined with heterologous DNA, and wherein said recombinant DNA is devoid of any DNA region involved in  
35       the biosynthesis of Bialaphos, when said fragment originates itself from a Bialaphos-producing strain.

11. The process according to claim 10, wherein the recombinant DNA is a vector suitable for the transformation of a cellular host and, when introduced therein, for allowing for the neutralization or inhibition of glutamine synthetase inhibitors.

12. The process according to claim 11, wherein the vector comprises said recombinant DNA under the control of replicon elements suitable for the transformation of bacteria, particularly E.coli.

13. The process of claim 11, wherein the vector comprises said recombinant DNA fragment under the control of a plant promotor region, which vector further comprises regulation elements allowing for the expression of said DNA fragments in plant cells, when the latter are later transformed with said vector.

14. The process of any of claims 10 to 13, which confers resistance against herbicidal effects of Bialaphos, PPT or related derivatives to the transformed plant cells.

15. The process of claim 13 or 14, wherein the vector comprises a nucleotide sequence encoding a transit peptide intercalated between said plant promotor region and said recombinant DNA fragment.

16. The process of claim 15, wherein the transit peptide is selected from ribulose-1,5 biphosphate carboxylase and chlorophyl a/b binding proteins.

17. The process of any of claims 11 to 16, wherein said vector is a Ti plasmid.

18. Plant cells, non biologically transformed, wherein they possess, stably integrated in their genome, a DNA fragment encoding a protein having a non-variety-specific enzymatic activity capable of neutralizing or inactivating glutamine synthetase inhibitors.

19. Plant cells according to claim 18, wherein they are capable of generating a plant capable of

producing seeds, said seeds having a non-variety-specific enzymatic activity capable of inactivating or neutralizing glutamine synthetase inhibitors.

5           20. Plant cells according to claim 18 or 19, wherein they are transformed by the process of any of claims 9 to 17.

          21. Plant cells according to any of claims 18 to 20, which produce detectable amounts of phosphinotricin acetyl transferase.

10           22. Seeds, non biologically transformed, which possess, stably integrated in their genome, a DNA fragment encoding a protein having a non-variety-specific enzymatic activity capable of inactivating or neutralizing glutamine synthetase inhibitors.

15           23. Seeds according to claim 22, which are capable of germinating into a plant capable of producing seeds having a non-variety-specific enzymatic activity capable of inactivating or neutralizing glutamine synthetase inhibitors.

20           24. Seeds according to claim 22 or 23, which are transformed by the process of any one of the claims 9 to 17.

          25. Plants, non biologically transformed, which possess, stably integrated in their genome, a DNA fragment encoding a protein having a non-variety-specific enzymatic activity capable of neutralizing or inactivating glutamine synthetase inhibitors.

25           26. Plants according to claim 25, which are capable of producing seeds having a non-variety specific enzymatic activity capable of inactivating or neutralizing glutamine synthetase inhibitors.

30           27. Plants according to claim 25 or 26, which are transformed by the process of any of claims 9 to 17.

          28. Process for protecting a plant species and  
35 selectively destroying weeds in a field comprising the

steps of treating a field with an herbicide, wherein the plant species contain in their genome a DNA fragment encoding a protein having an enzymatic activity capable of neutralizing or inactivating glutamine synthetase inhibitors, and wherein the used herbicide is a glutamine synthetase inhibitor.

29. Process according to claim 28, wherein the plant species contains a DNA fragment according to any one of claims 1 to 8.

30. Process according to claim 28 or 29, wherein the plant species are transformed according to the process of any one of claims 9 to 17.

31. Process according to any one of claims 28 to 30, wherein a solution of a glutamine synthetase inhibitor is applied on the field, after emergence of the cultivated plant species, several times, particularly at time intervals of about 20 to 100 days, until early and late germinating weeds are destroyed.

32. Process according to any of claims 28 to 31, wherein glutamine synthetase inhibitors comprise Bialaphos, phosphinotricin and related compounds.

33. Process for selectively protecting a plant species in a field against fungal diseases comprising the steps of treating a field with an herbicide, wherein the plant species contain in their genome a DNA fragment encoding a protein having an enzymatic activity capable of neutralizing an inactivating glutamine synthetase inhibitors and wherein the used herbicide is a glutamine synthetase inhibitor.

34. Process according to claim 33, wherein the plant species contain a DNA fragment according to any of claims 1 to 8.

35. Process according to claim 33 or 34, wherein the plant species are transformed according to the process of any one of claims 9 to 17.

36. Process according to any one of claims 33 to 35, wherein a solution of a glutamine synthetase inhibitor is applied on the field, after emergence of the cultivated plant species, several times, particularly at \_\_\_\_\_ time intervals of about 20 to 100 days until fungi are destroyed.

37. Process according to any one of claims 33 to 36, wherein glutamine synthetase inhibitor is selected from a group which comprises Bialaphos, phosphinotricin and related compounds.

38. Process according to claim 32 or 37, wherein Bialaphos, PPT or related compound is applied at a dose ranging from about 0.4 to about 1.6 kg/ha.

39. Process according to claim 38, wherein Bialaphos, PPT or related compound is diluted in a liquid carrier at a concentration such as to enable its application to the field at a rate ranging from about 2 l/ha to about 8 l/ha.

40. Process according to any of claim 38 or 39, wherein selectively protected plant species comprise sugar-beet, rice, potato, tomato, maize, tobacco.

41. Vector which contains a DNA fragment encoding a polypeptide having an enzymatic activity capable of causing inactivation of glutamine synthetase inhibitors.

42. Vector according to claim 41, which is suitable for the transformation of plant cells and plants.

43. Vector according to claim 41 or 42, which contains a DNA fragment belonging to the genome of a Streptomyces.

44. Vector according to any of claims 41 to 43, which contains the DNA fragment recombined with heterologous DNA, wherein said recombinant DNA is devoid of any DNA region involved in the biosynthesis of Bialaphos.



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45. The vector of claim 44 suitable for the transformation of a cellular host and, when introduced therein, for allowing for expression of a polypeptide capable of inactivating glutamine synthetase inhibitor.

46. The vector of claim 45, wherein said DNA fragment is under the control of replicon elements suitable for the transformation of bacteria, particularly E. coli.

47. The vector of claim 46, wherein said DNA fragment is under the control of a plant promoter region, and of regulation elements allowing for the expression of said DNA fragments in plant cells, when the latter are later transformed with said vector.

48. The vector of claim 47, which confers bialaphos-resistance to the transformed plant cells.

49. The vector of claim 48, wherein a sequence encoding a transit peptide is intercalated between said plant promoter region and said DNA fragment.

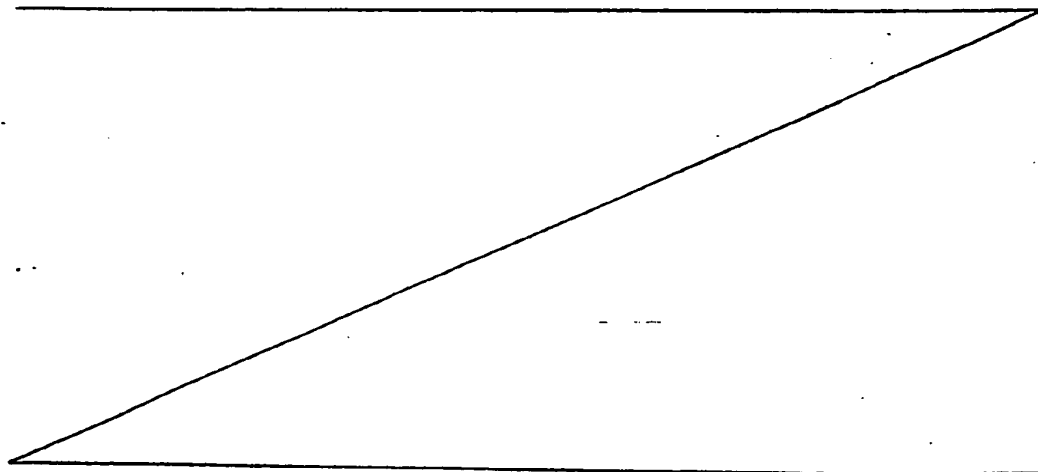
50. The vector of claim 49, wherein the transit peptide is selected from ribulose-1,5 biphosphate carboxylase and chlorophyl a/b binding proteins.

51. The vector of any of claims 45 to 50, wherein said vector is a modified Ti plasmid.

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52. Vector according to any of claims 41 to 51,  
which comprises the following nucleotide sequence :

GTG AGC CCA GAA

5      183 . . . . .

CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG  
229

GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC  
273

10    AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC  
318

CTC GTC CGT CTG CCG GAG CCG TAT CCC TGG CTC GTC GCC GAG GTG  
363

GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA  
408

15    CCG AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC  
453

CCC CSC CAC CAG CCG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC  
498

20    CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT  
543

GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CCG ATG CAC GAG GCG  
588

CTC GGA TAT GCC CCC CCG GGC ATG CTG CCG GCG GCC GGC TTC AAG  
633

25    CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC  
678

CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC  
723

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53. The vector of claim 52, wherein the initiation codon ATG is substituted for the initiation codon GTG of the sequence defined in claim 52.

5 54. Vector according to any of claims 41 to 51, which comprises the following nucleotide sequence :

TAAAGAGGTGCCCGCCACCCGCTTTCGCAGAACACCGAAGGAGACCACAC  
↓  
10 GTGAGCCCAGAACGACGCCCGGTCTGAGATCCGTCCCGCCACCGCCGCCGA  
CATGGCGGCGGTCTGCGACATCGTCAATCACTACATCGAGACGAGCACGG  
TCAACTTCCGTACGGAGCCGCAGACTCCGCAGGAGTGGATCGACGACCTG  
15 GAGCGCCTCCAGGACCGCTACCCCTGGCTCGTCGCCGAGGTGGAGGGCGT  
CGTCGCCGGCATCGCCTACGCCGGCCCCCTGGAAGGCCCGCAACGCCTACG  
ACTGGACCGTCGAGTCGACGGTGTACGTCTCCACCGGCACCAGCGGCTC  
20 GGAETGGGCTCCACCCTCTACACCCACCTGCTGAAGTCCATGGAGGCCCA  
GGGCTTCAAGAGCGTGGTCTGCCGTTCATCGGACTGCCCAACGACCCGAGCG  
25 TGCGCCTGCACGAGGCGCTCGGATACACCGCGCGCGGGACGCTGCGGGCA  
GCCGGCTACAAGCACGGGGGGCTGGCACGACGTGGGGTTCTGGCAGCGCGA  
CTTCGAGCTGCCGGCCCCGCCCCGCCCCGTCCGGCCCGTCACACAGATCT  
30 GAGCGGAGAGCGCATGGC ↑

35

72

55. A DNA fragment, for the subsequent transformation of plant cells, coding for a polypeptide having phosphinothricin-acetyl-transferase activity, which consists of a nucleotide sequence coding for the following amino acid sequence :

I SER PRO GLU

123  
ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO  
10 229  
ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL  
273  
ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP  
318  
15 LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL  
363  
ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA  
408  
ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER  
20 453  
PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS  
498  
LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA  
543  
25 VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA  
588  
LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS  
633  
HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER  
30 678  
LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE

723

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in which X represents MET or VAL, or of a part of said nucleotide sequence of sufficient length to code for a polypeptide still having phosphinotricin-acetyl-transferase activity.

- 5 56. The DNA fragment of claim 55 which comprises the following nucleotide sequence :

```

183      GTG AGC CCA GAA
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
10 228
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
273
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
318
15 CTC GTC CGT CTG CCG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
363
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
408
CSC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
20 453
CCC CGC CAC CAG CCG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
498
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
543
25 GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
588
CTC GGA TAT GCC CCC CGC GGC ATG CTG CCG GCG GCC GGC TTC AAG
633
CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC ASC
30 678
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
723

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74

or a part thereof expressing a polypeptide having phosphinotricin-acetyl-transferase activity.

57. A DNA fragment, for the subsequent transformation of plant cells, coding for a polypeptide having phosphinotricin-acetyl-transferase activity, which consists of a nucleotide sequence coding for the following amino acid sequence :

10 V S P E R R P V E I R P A T A A D M  
A A V C D I V N H Y I E T S T V N F  
15 R T E P Q T P Q E W I D D L E R L Q  
D R Y P W L V A E V E G V V A G I A  
20 Y A G P W K A R N A Y D W T V E S T  
V Y V S H R H Q R L G L G S T L Y T  
25 H L L K S M E A Q G F K S V V A V I  
G L P N D P S V R L H E A L G Y T A  
30 R G T L R A A G Y K H G G W H D V G  
F W Q R D F E L P A P P R P V R P V  
35 T Q I \*

or of a part of said nucleotide sequence of sufficient length to code for a polypeptide still having phosphotransferase activity.

5 58. The DNA fragment of claim 57, which comprises the following nucleotide sequence :

```
TAAAGAGGTGCCCGCCACCCGCTTTCGCAGAACACCGAAGGAGACCACAC
↓
10 GTGAGCCCAGAACGACGCCCGGTTCGAGATCCGTCCCGCCACCGCCGCCGA

CATGGCGGCGGTCTGCGACATCGTCAATCACTACATCGAGACGAGCACGG

TCAACTTCCGTACGGAGCCGCAGACTCCGCAGGAGTGGATCGACGACCTG

15 GAGCGCCTCCAGGACCGCTACCCCTGGCTCGTCGCCGAGGTGGAGGGCGT

CGTCGCCGGCATCGCCTACGCCGGCCCCCTGGAAGGCCCGCAACGCCTACG

ACTGGACCGTCGAGTCGACGGTGTACGTCTCCACCGGCACCAGCGGCTC

20 GGACTGGGCTCCACCCTCTACACCCACCTGCTGAAGTCCATGGAGGCCCA

GGGCTTCAAGAGCGTGGTCGCCGTCATCGGACTGCCCAACGACCCGAGCG

25 TGCGCCTGCACGAGGCGCTCGGATACACCGCGCGGGACGCTGCGGGCA

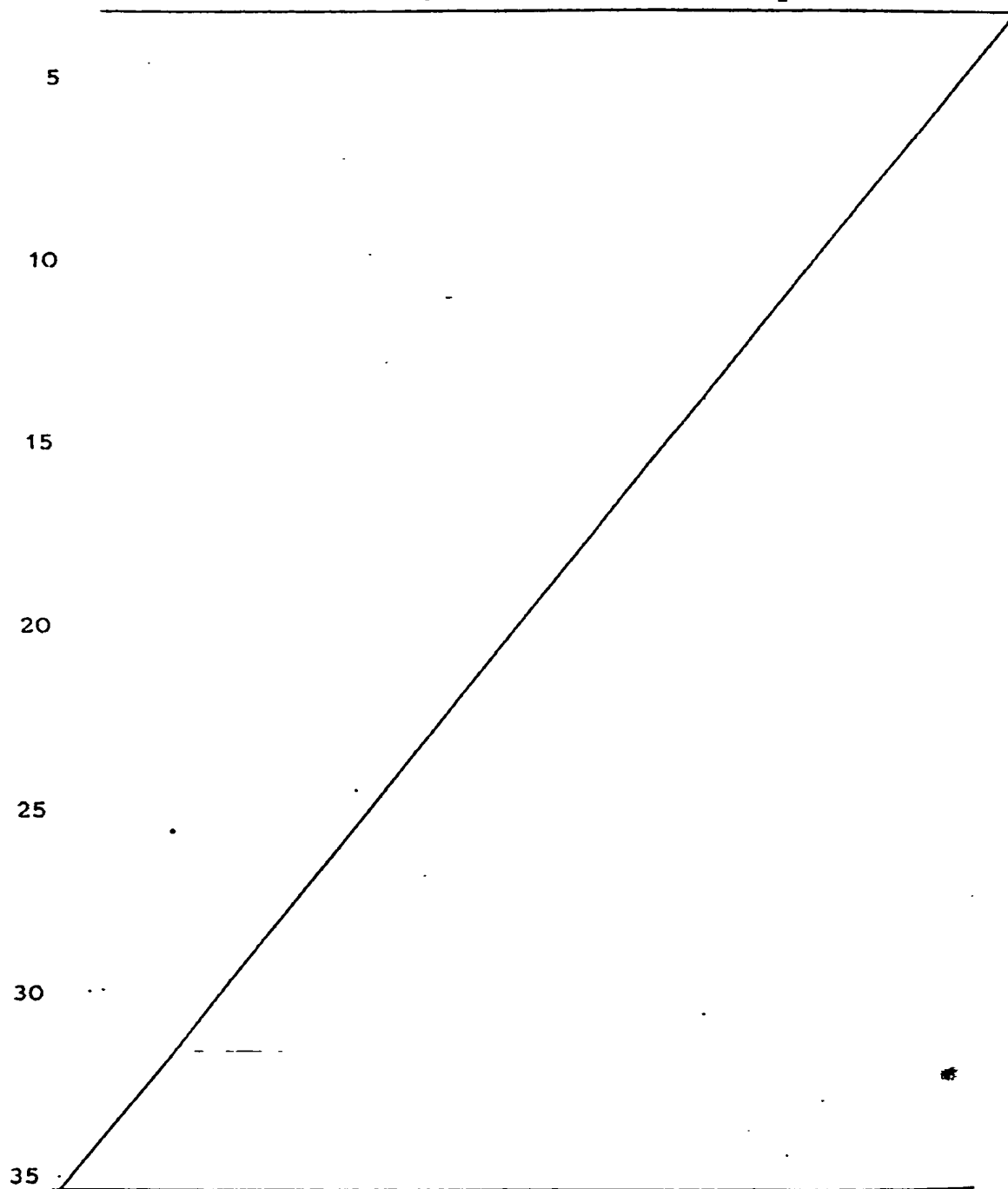
GCCGGCTACAAGCACGGGGGCTGGCACGACGTGGGGTTCTGGCAGCGCGA

CTTCGAGCTGCCGGCCCCGCCCGCCCCGTCCGGCCCGTCACACAGATCT
30 GAGCGGAGAGCGCATGGC
```

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or of a part thereof expressing a polypeptide having phosphinotricin-acetyl-transferase activity.





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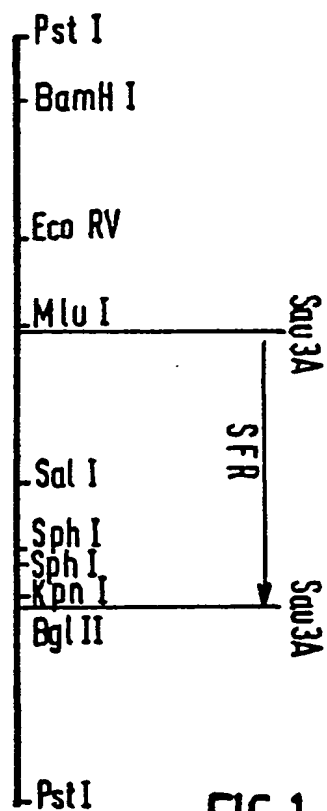


FIG. 1

ERSATZBLATT

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CCC GCT CAA GCT CGC TGT CAT TTT CGA GAC GCC ATC TTT GGA AGC  
 GGT GGC CGA ATC CGT ACT GCG CGG ACT CGA CGA CGC GTA AAA CGA  
 TCG ACC ACG TAC ACG AGT CCG GAC ACG GGG CGA GGA GGC CCG GTT  
 CCG GCA CCG AGG AAG ACC GAA GGA AGA CCA CAC GTG AGC CCA GAA  
 CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG  
 FokII  
 GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC  
 AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC  
 CTC GTC CGT CTG CCG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG  
 GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA  
 CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC  
 CCC CGC CAC CAG CCG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC  
 CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT  
 GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG  
 CTC GGA TAT GCC CCC CGC GGC ATG CTG CCG GCG GCC GGC TTC AAG  
 CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC  
 CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC TGA  
 ACG GAG TGC GCG TGG GCA TCG CCC GAG TTG GAG CTG GTA CCG GAA  
 CTC ATC GAA CTC AAC TGG CAT ACC CGC AAT GGT GAG GTG GAA CCG  
 CCG CCG ATC GCG TAC GAC CGT GCC CAG G

FIG. 2

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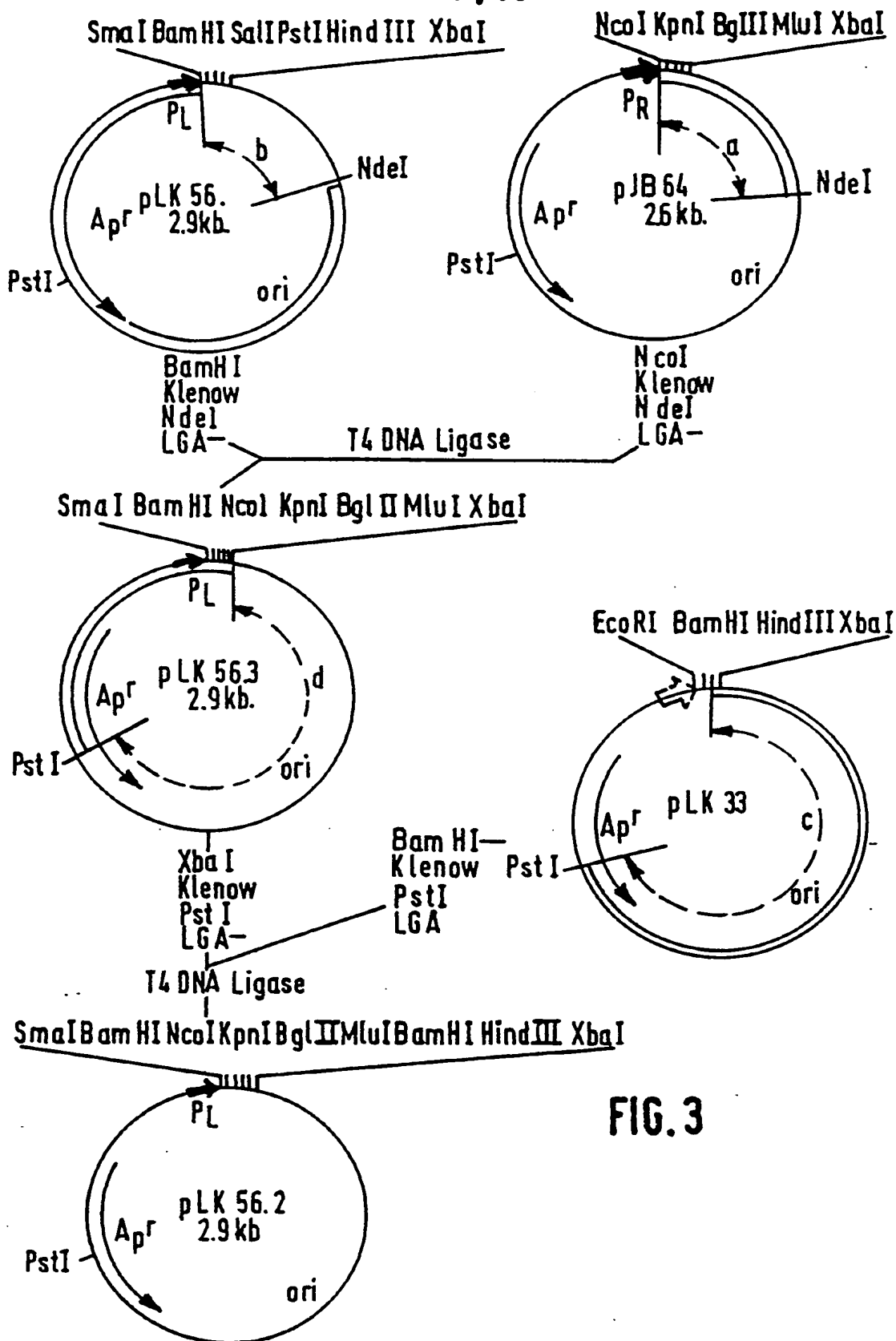


FIG. 3

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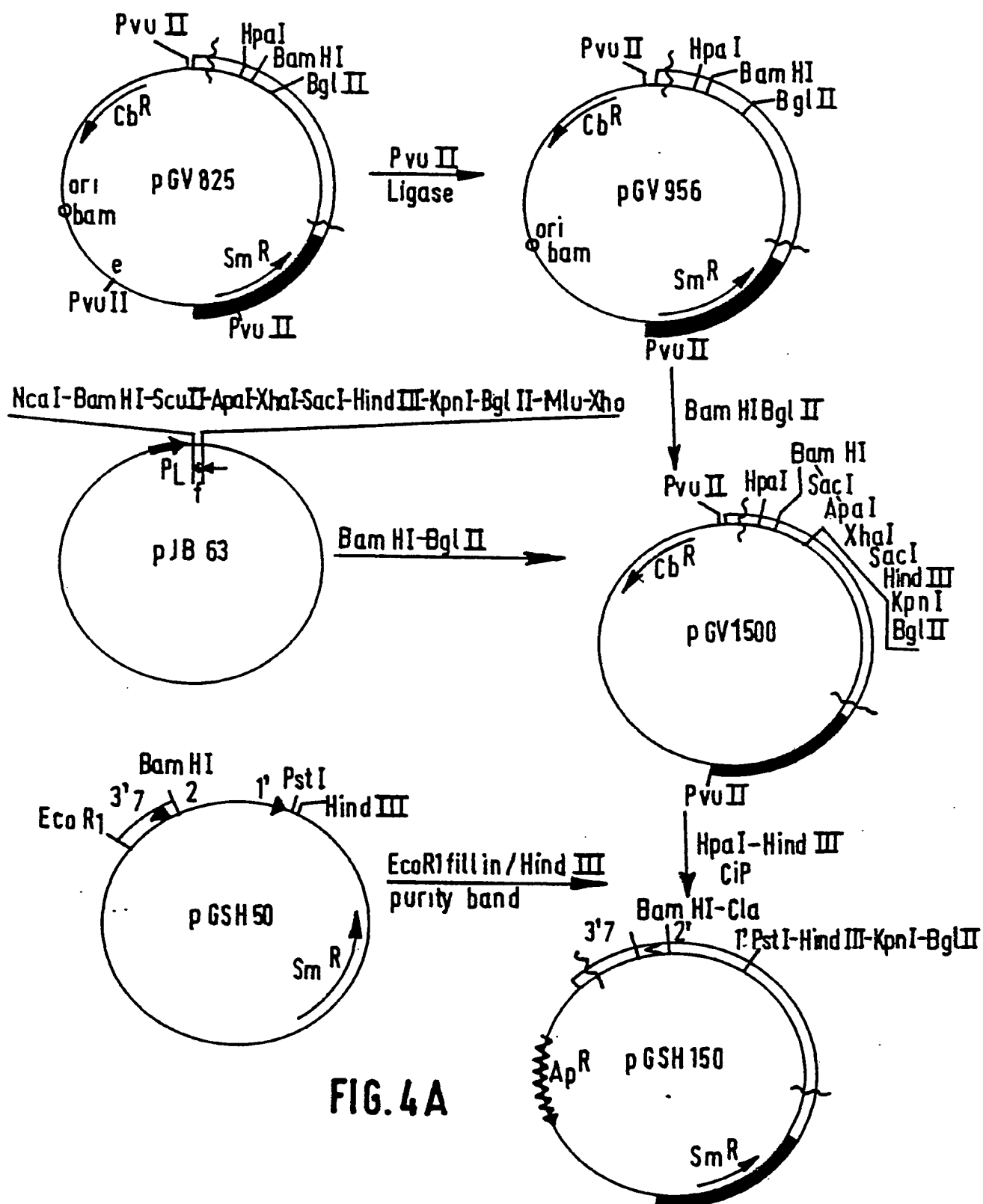


FIG. 4A

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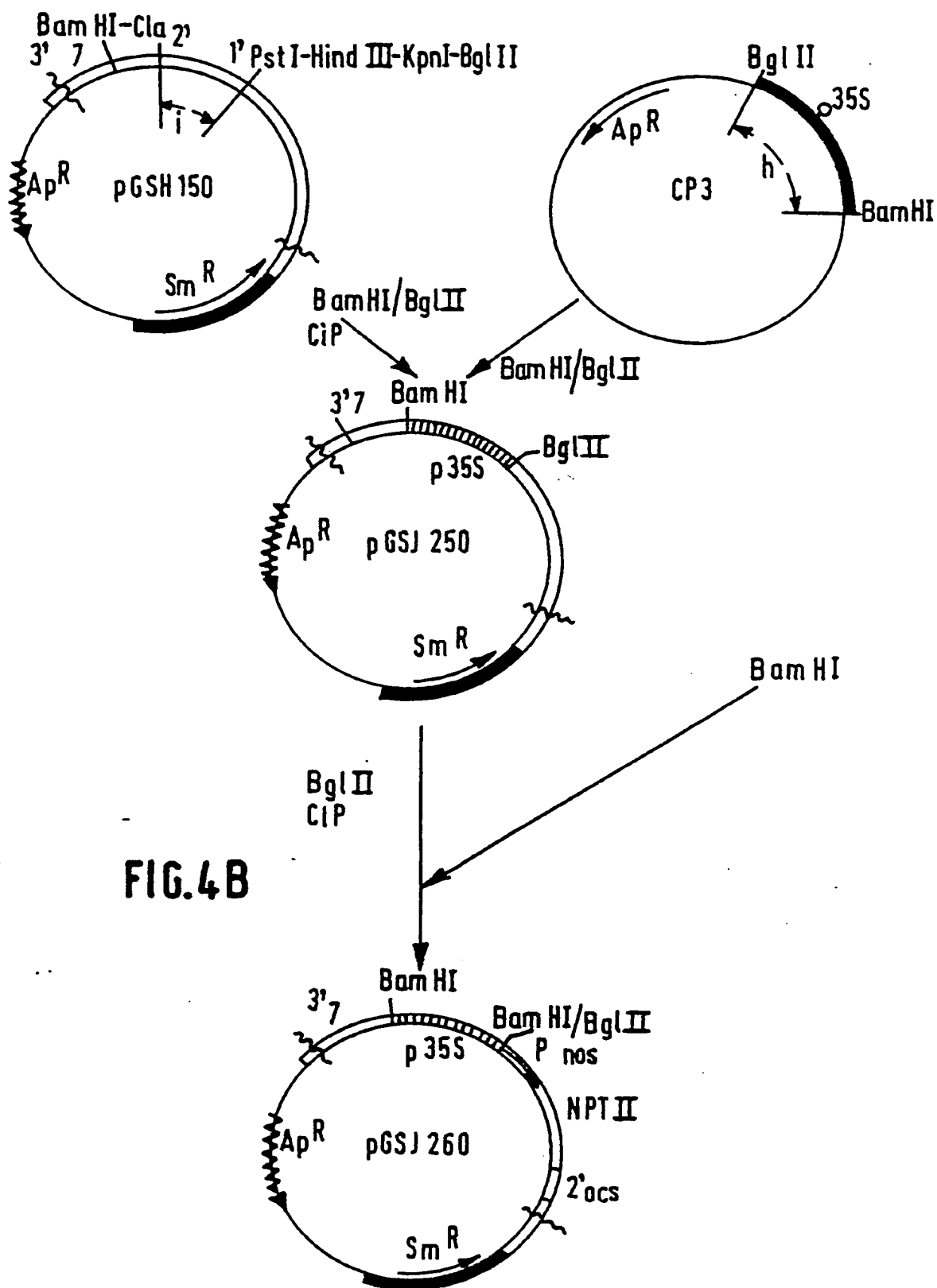


FIG. 4B

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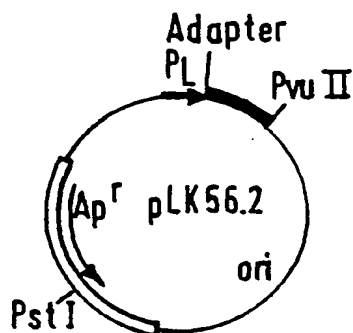


FIG. 5A

Nco I / Bgl II

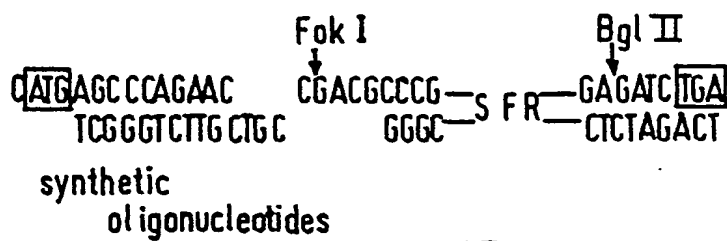


FIG. 5B

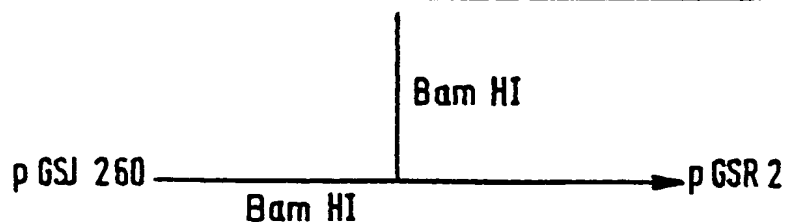
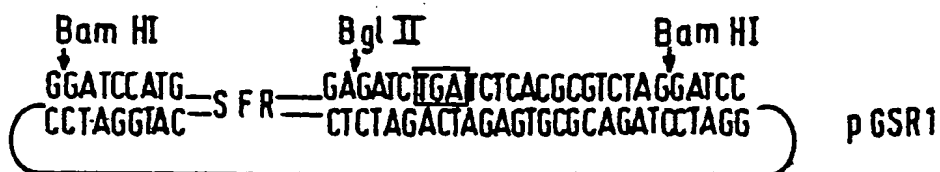
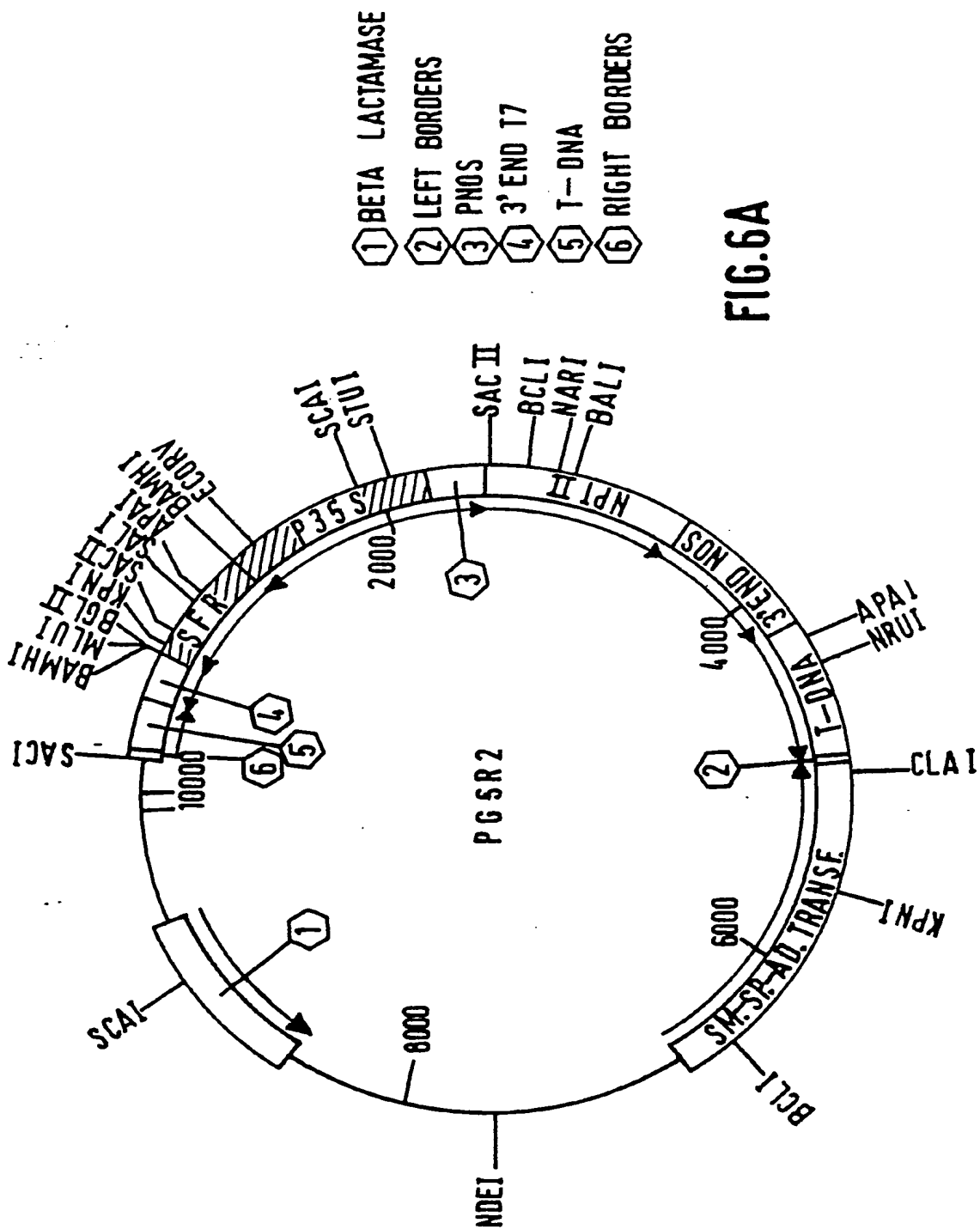


FIG. 5C

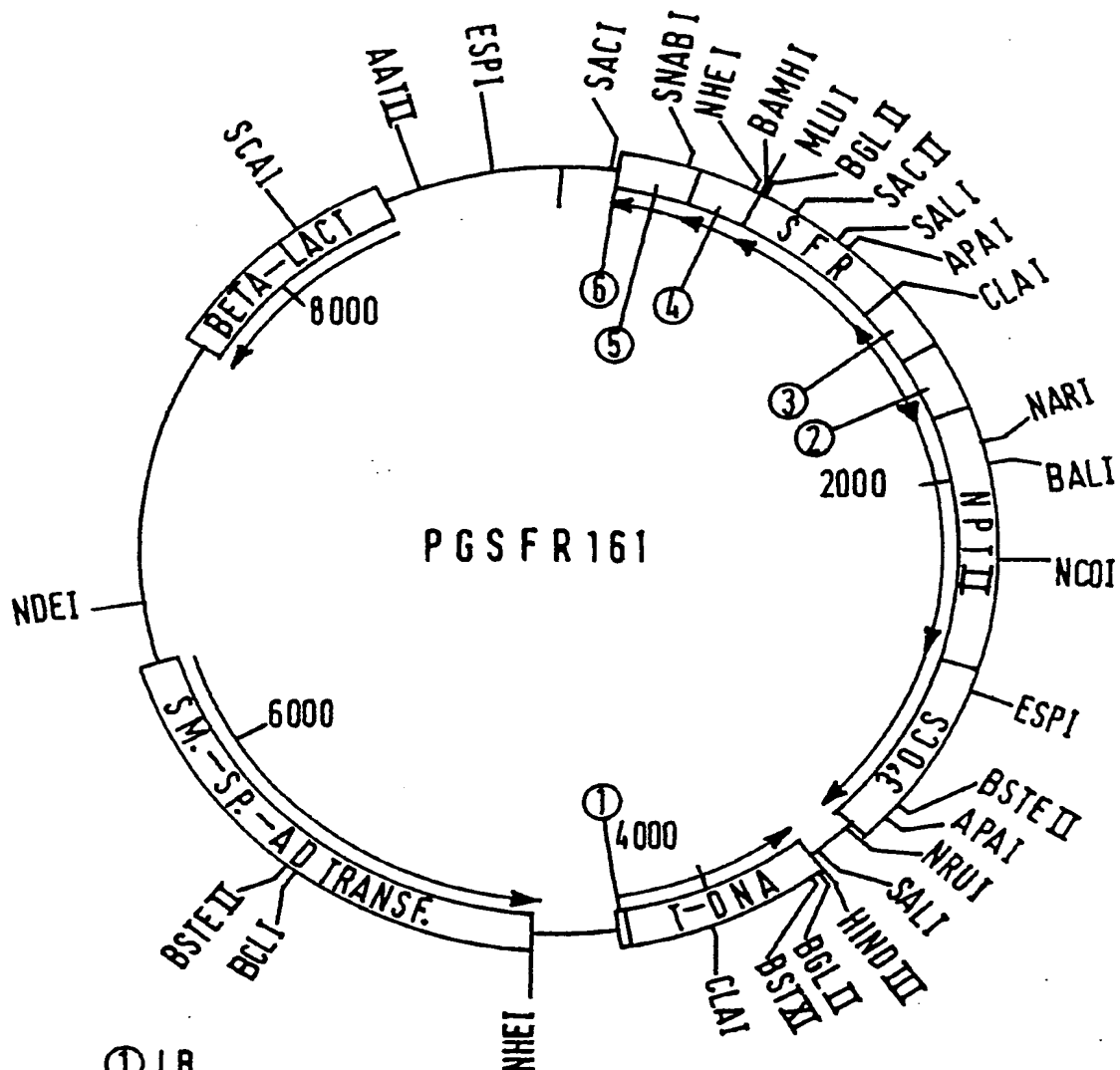
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**FIG. 6A**

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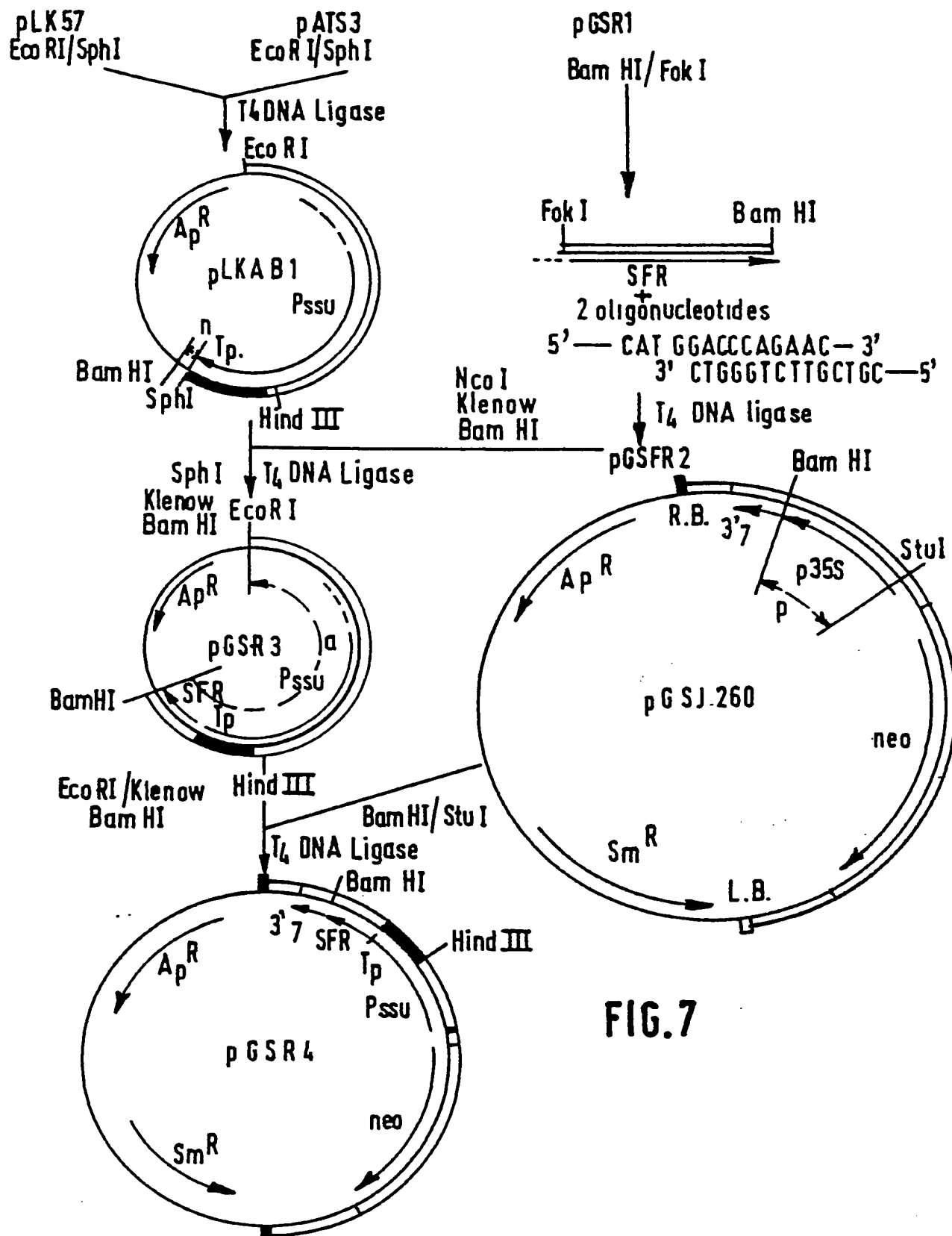
- ① LB
- ② PTR1
- ③ PTR2
- ④ 3'END7
- ⑤ T-DNA
- ⑥ R.B.

FIG.6B

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**FIG. 7**

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**Fig. 8**

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TAAAGAGGTG C C C G C C A C C C G C T T T C G C A G A A C A C C G A A G G A G A C C A C A C  
↓  
G T G A G C C C A G A A C G A C G C C C G G T C G A G A T C C G T C C C G C C A C C G C C G C C G A  
C A T G G C G G C G G T C T G C G A C A T C G T C A A T C A C T A C A T C G A G A C G A G C A C G G  
T C A A C T T C C G T A C G G A G C C G C A G A C T C C G C A G G A G T G G A T C G A C G A C C T G  
G A G C G C C T C C A G G A C C G C T A C C C C T G G C T C G T C G C C G A G G T G G A G G G C G T  
C G T C G C C G G C A T C G C C T A C G C C G G C C C C T G G A A G G C C C G C A A C G C C T A C G  
A C T G G A C C G T C G A G T C G A C G G T G T A C G T C T C C C A C C G G C A C C A G C G G C T C  
G G A C T G G G C T C C A C C C T C T A C A C C A C C T G C T G A A G T C C A T G G A G G C C C A  
G G G C T T C A A G A G C G T G G T C G C C G T C A T C G G A C T G C C C A A C G A C C C G A G C G  
T G C G C C T G C A C G A G G C G C T C G G A T A C A C C G C G C G C G G G A C G C T G C G G G C A  
G C C G G C T A C A A G C A C G G G G G C T G G C A C G A C G T G G G G T T C T G G C A G C G C G A  
C T T C G A G C T G C C G G C C C G C C C C G C C C C G T C C G G C C C G T C A C A C A G A T C T  
↑  
G A G C G G A G A G C G C A T G G C

FIG.9

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10 20 30 40 50  
 PBG39----- MSPERRPADI RRATEADMPA VCTIVNHYIE TSTVNFRTPE QEPQEWTDOL  
 PJS1----- VSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTPE QTPQEWTDOL

60 70 80 90 100  
 PBG39----- VRLRERYPWL VAEVDGEVAG IAYAGPWKAR NAVDWTAESE VVSPRHHQRT  
 PJS1----- ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAVDWTVEST WVSHRHQRL

110 120 130 140 150  
 PBG39----- GLGSTLYTHL LKSLEAQQGFK SVVAVIGLPN DPSVRMHEAL GVAPRGMMLRA  
 PJS1----- GLGSTLYTHL LKSMEAQQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA

160 170 180  
 PBG39----- AGFKHGNWHD VGFWQLDFSL PVPPRPVLPV TEI\*  
 PJS1----- AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV IQI\*

FIG.10

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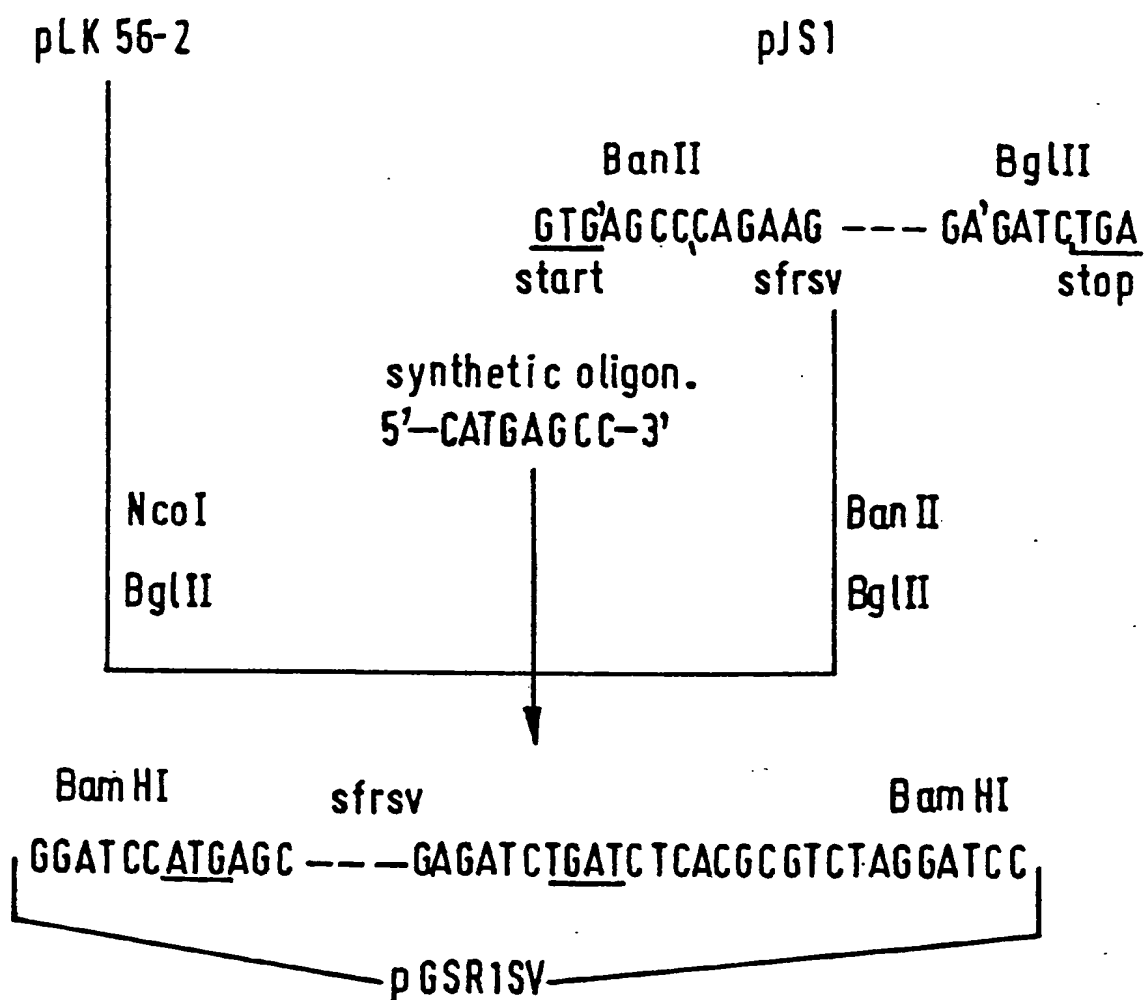


FIG. 11

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# INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 87/00141

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> C 12 N 15/00; A 01 H 1/00; C 12 N 5/00; IPC: A 01 H 5/10; // C 12 N 9/10																							
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; text-align: left; border-bottom: 1px solid black;">Classification System</th> <th style="text-align: left; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC<sup>4</sup></td> <td style="padding: 5px;">C 12 N; A 01 H; A 01 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched<sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>4</sup>	C 12 N; A 01 H; A 01 N																	
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IPC <sup>4</sup>	C 12 N; A 01 H; A 01 N																						
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; text-align: center;">Category<sup>9</sup></th> <th style="width: 60%; text-align: center;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 30%; text-align: center;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;">EP, A, 0173327 (MEIJA SEIKA KAISHA) 5 March 1986 see page 19, lines 28-36; figure 7</td> <td style="vertical-align: top;">12, 41, 43, 45, 46, 52, 55, 56 1-40, 42, 44, 47-51, 53, 54, 57, 58</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="text-align: center; vertical-align: top;">--</td> <td></td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">WO, A, 84/02920 (MONSANTO) 2 August 1984 see page 12, lines 13-18; page 39, lines 1-16; claims 29-34</td> <td style="vertical-align: top;">1-58</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Journal of Molecular and Applied Genetics, volume 2, no. 6, 1984, Raven Press, (New York, US), G. Donn et al.: "Herbicide-resistant alfalfa Cells: An example of gene amplification in plants", pages 621-635 see page 625, right-hand column, last paragraph</td> <td style="vertical-align: top;">1-58</td> </tr> <tr> <td></td> <td style="text-align: center;">--</td> <td></td> </tr> <tr> <td></td> <td style="text-align: right;">./.</td> <td></td> </tr> </tbody> </table>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	EP, A, 0173327 (MEIJA SEIKA KAISHA) 5 March 1986 see page 19, lines 28-36; figure 7	12, 41, 43, 45, 46, 52, 55, 56 1-40, 42, 44, 47-51, 53, 54, 57, 58	Y	--		Y	WO, A, 84/02920 (MONSANTO) 2 August 1984 see page 12, lines 13-18; page 39, lines 1-16; claims 29-34	1-58	Y	Journal of Molecular and Applied Genetics, volume 2, no. 6, 1984, Raven Press, (New York, US), G. Donn et al.: "Herbicide-resistant alfalfa Cells: An example of gene amplification in plants", pages 621-635 see page 625, right-hand column, last paragraph	1-58		--			./.	
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Y	--																						
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																							
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">4th July 1987</td> <td style="border-bottom: 1px solid black; padding: 5px;">12 AUG 1987</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="padding: 5px;">M. VAN MOL </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	4th July 1987	12 AUG 1987	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	M. VAN MOL													
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EUROPEAN PATENT OFFICE	M. VAN MOL																						

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, volume 317, 24 October 1985, R. Shields: "Engineering herbicide resistance", page 668 see the whole article --	1-17, 47-51
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Y	The Pesticide Manual, A World Compendium, 7th edition, 1983, ed. by Charles R. Worthing, published by The British Crop Protection Council, (London, GB), see page 302, no. 6930 --	28-40
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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A	-- Proceedings of the Nationale Academy of Sciences of USA, volume 81, May 1984, A.R. Cashmore: "Structure and expression of a pea nuclear gene encoding a chlorophyll a/b-binding polypeptide", pages 2960-2964 see page 2964	13-17,47-51
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A	-- Nucleic Acids Research, volume 14, no. 4, February 1986, IRL Press Limited, (Oxford, GB), M. Zalacain et al.: "Nucleotide sequence of the hygromycin B phospho- transferase gene from Streptomyces hygroscopicus", pages 1565-1581, see page 1573, paragraph 1; page 1577, paragraph 2	6,53
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## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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A	GB, A, 2007976 (MEIJI SEIKA) 31 May 1979 -----	

# ANNEX THE INTERNATIONAL SEARCH REPORT

INTERNATIONAL APPLICATION NO.

PCT/EP 87/00141 (SA 16647)

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		AU-A- 4130978	17/05/79
		AU-B- 514686	19/02/81
		CA-A- 1120280	23/03/82
		DE-C- 2857693	09/10/86
		FR-A, B 2407671	01/06/79
		JP-A- 54119034	14/09/79
		US-A- 4309208	05/01/82
		US-A- 4448601	15/05/84
		US-A- 4455163	19/06/84
		US-A- 4552584	12/11/85
		US-A- 4622060	11/11/86

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